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**A STUDY OF THE IN VITRO CULTURE OF
ERYTHROPOIETIC TISSUE**

CLIFTON L. NELSON

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THE UNIVERSITY OF ALBERTA

A STUDY OF THE IN VITRO CULTURE OF

ERYTHROPOIETIC TISSUE

A DISSERTATION

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ABSTRACT

A project has been outlined in which bone marrow cultures are proposed as a biological assay material for the detection of a possible circulating erythropoietic inhibitor produced by malignant cells. An erythropoietic inhibitor has been postulated as one of the causes of anemia frequently associated with cancer.

A review of the incidence and causes of anemia in cancerous patients and in tumor-bearing animals has been presented. The various methods of bone marrow culture have also been reviewed. The technique of Osgood and Brownlee was chosen because of its relative simplicity and good physiological conditions.

The present day concepts of erythropoiesis have been summarized.

The technique of the above method as used and the results of 101 separate bone marrow cultures are presented. It is concluded that short term (6 hours) cultures are suitable for this project and that a culture medium of Gey's fluid fortified with homologous serum offers satisfactory growth.

Suggestions are offered for improving the growth responses in cultures, based upon experience and information obtained in the past year.

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SECTION I

INTRODUCTION

Anemia has been widely recognized by clinicians as a frequent concomitant of malignancy. In some cases this anemia is readily accounted for by such obvious causes as blood loss, dietary deficiency, or replacement of hemopoietic bone marrow by metastases. In other cases where there is no explanation for the anemia, the presence of an erythropoietic inhibitor released by malignant cells has been postulated.

The formation of such an inhibitor substance by malignant cells might be expected to result in its presence in the blood stream. It would thus have ready

access to the bone marrow where it might exert its inhibitory influence which would then account for the anemia. Conversely, the development of anemia in experimental animals exposed to extracts of blood or urine (presuming that the inhibitor might be excreted in the urine) from cancerous patients would indicate the presence of such a substance. Alternatively, bone marrow cultures might be exposed to extracts of blood or urine from cancerous patients, and their hemopoietic influences compared with those of similar extracts from normal individuals.

It is a primary purpose of this project to investigate by the simplest possible means the possibility of an erythropoietic inhibitor. The complexity of the field of hemopoiesis makes it essential that this study be purposefully restricted in its technical scope. If a relatively simple and rapid method for the detection of an inhibitor could be developed, it would have immediate practical application in the diagnosis of malignancy. A complicated or prolonged procedure would make such application impracticable. Therefore even if an "in vivo" means should detect an inhibitor, the time lapse involved would reduce the practical value of the procedure as a diagnostic aid. The first plan mentioned above was therefore rejected.

Even in considering the second alternative, that of bone marrow cultures, difficulties were foreseen.

There are many methods of bone marrow culture which could be used and each would have to be evaluated in order that the one most suitable for the problem would be chosen. The techniques and methods of culturing bone marrow cells are not widely used and delay would result in learning and standardizing the technique of the chosen method. Errors in the cell counts used to determine the amount of growth would have to be determined so that the reliability of the method as a whole could be known. However, once these preliminary problems had been solved, the cultures, because of their rapid growth, should provide a quick means of determining the presence of an inhibitor if they are sensitive enough.

The project which was set up, therefore, proposed the use of bone marrow cultures as the biological assay method to attempt to prove the presence of an erythropoietic inhibitor released by malignant cells. The first phase of the project thus involved the determination of the accuracy of the cell counts used in following the growth in the cultures. The second phase, and the one with which this thesis will be largely concerned, was the culturing of bone marrow cells themselves in order that the technique might be learned and standardized. The third phase of the project deals with the testing of various extracts of urine and serum from patients with malignant disease for their effect on these cultures.

SECTION II

REVIEW OF THE RELEVANT LITERATURE

A. Anemia in Cancerous Patients

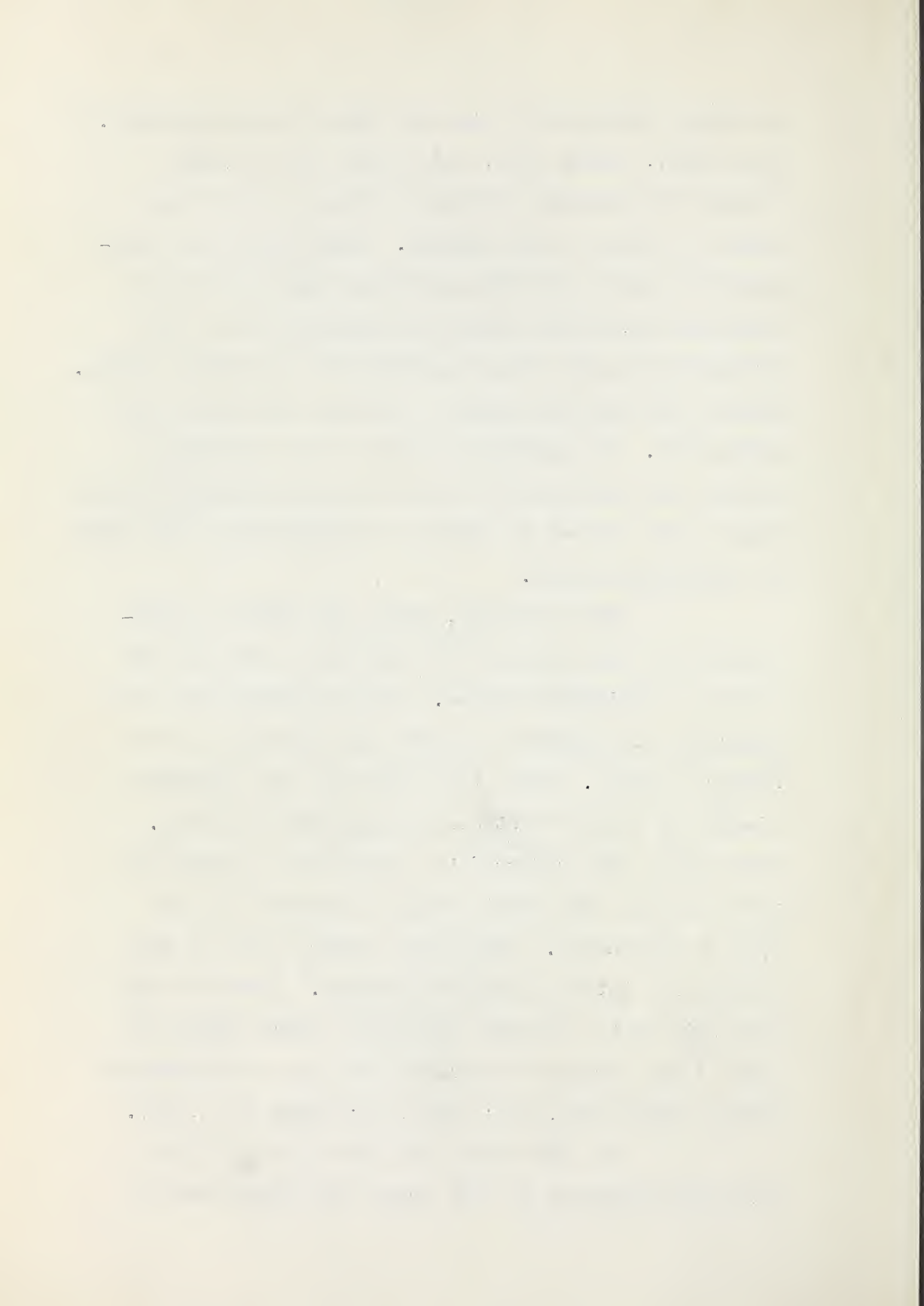
1. Occurrence and Incidence of this Anemia

For many years clinicians have reported a frequent association of anemia with malignancy. Ewing (52) referred to the work of Louis in 1846 as showing that there is a diminished quantity of blood in malignancy. In 1886 Eichhorst (42) stated with respect to cancer of the stomach, "The blood is often very pale, almost serous, and poor in red blood-globules." In reference to cancer of the liver (43) he stated, "On examination of the blood, it is often found that the red blood-globules are scanty, very pale and present

striking variations in size and shape (poikilocytosis)." Osler (98), Gibson (57), and Allbutt (3) in their respective textbooks mentioned anemia as one of the signs of cancer of the stomach. Osler called it a progressive anemia and while admitting that it sometimes resembles pernicious anemia he observed that it more frequently has the characteristics of a secondary anemia. Gibson (58) noticed anemia in patients with pancreatic malignancy. In speaking of cancer of the esophagus Allbutt and Rolleston (4) stated that the apparent anemia seen in the patient is masked on examination of the blood by hemoconcentration.

More recently, Ewing (51) found a deterioration in both quality and quantity of blood in the course of malignant disease. He also noticed that the reduction of hemoglobin exceeds the reduction in the number of cells. Haden (67) observed that malignant disease is almost constantly accompanied by anemia. Clark et al (29) referred to a statement of Krache in which he said that anemia could be produced by any type of malignancy. Many other authors refer to the association between anemia and cancer. Indeed it has been said that a diligent search for cancer should be made in any person of middle age who has an unexplained anemia (Schwertman, 114; Cecil, 28; Brown et al, 20).

In 1928 Eisen (46) made a review of 353 cases of malignancy in many organs and found the red



cell count averaged 3,569,000, the hemoglobin 62%, and the color index 0.88. Morrison in 1932 (79) reviewed 100 cases of cancer in various organs and noted that 43% had a hemoglobin of 50% or below, 25% had hemoglobins between 51% and 64% and only 32% had hemoglobins of 65% or above. In 13% of the cases the red count was below 3,000,000, in 64% between 3,000,000 and 5,000,000 and in only 23% above 5,000,000. He concluded that in malignancy the reduction in erythrocytes is preceded by a reduction in the hemoglobin and depending on the balance of the two, the color index may be high or low. In 1947 Clark et al (29) analysed 38 patients with malignant disease from the standpoint of blood volume and chronic shock. They concluded that the major factor responsible for the reduced blood volume associated with malignant disease is a deficiency of the total circulating cell mass and hemoglobin. Schwertman (114) examined 58 cases of carcinoma in 8 different organs in 1948 and found anemia, chiefly of the hypochromic type, to be present in all. In contrast to this, Bateman (12) in 1951 found the blood volume in 33 patients with cancer to be variable in malignant disease but he did report an associated anemia. Shen and Homberger (115) in 1951 studied 193 cases of advanced cancer and found that 60.1% had a hemoglobin below 80%. Totterman (125) makes mention of 14 cases of cancer in

different sites uncomplicated by hemorrhage or infection in which the average hemoglobin was 70.4%.

2. Degree of Anemia in Cancer in Different Locations

Eisen (44) reported 16 cases of cancer in the oro-pharyngeal cavity in which the hemoglobin averaged 73% and the red cells 3,831,000. In the same report he also lists 13 cases of cancer of the esophagus with an average hemoglobin of 61% and average red cell count of 3,731,000. Master (77) reports a much higher average hemoglobin level and red cell count in 15 cases of squamous cell carcinoma of the esophagus, namely, 93% and 5,040,000. However, in 3 cases of adenocarcinoma of the same organ he found the hemoglobin averaged 46% and the red cell count 4,095,000.

There seems to be a good deal more in the literature dealing with anemia in association with gastric carcinoma than with its occurrence in cancer in any other site. Perhaps this is because of the relative frequency of gastric carcinoma. Master (77) reported 26 cases of carcinoma of the stomach in which the average hemoglobin was 58% and the average red cell count 3,370,000. Eisen (44) found that the hemoglobin averaged 50% and the red cell count 3,179,000 in 79 cases that he analyzed. Morrison observed (79) a lower hemoglobin in 22 cases, namely, an average of 38%. A much higher level, however, was found by Ariel (9) in a series of 79 cases. Here the hemoglobin averaged

70% and the red cells 3,500,000. Oppenheim (87) reported 122 cases and found that 64% had an abnormally low concentration of hemoglobin and low red cell counts. Clark et al (29) examined 10 patients with carcinoma of the stomach or lower portion of the esophagus from the standpoint of blood volume, finding the red cell mass averaged only 50% of normal and the hemoglobin mass 45% of normal.

Cancer of the colon is also associated with a severe degree of anemia. In his series of 187 patients in 1927, Eisen (44) includes 12 cases of cancer of the intestine, caecum and colon, in which the hemoglobin averaged 56% and the red cells 3,259,000. In 12 cases of cancer of the sigmoid colon he found an average hemoglobin of 68% and average red cell count of 3,841,000. Average levels of hemoglobin of 66% and red cell counts of 3,715,000 were found in 37 cases of rectal carcinoma. Morrison (79) reports 15 cases of cancer in the colon with an average hemoglobin of 50%. Alvarez et al (6) found anemia to be more severe in cancer of the caecum and ascending colon than in the transverse, descending and pelvic portions which showed a progressively more mild anemia. In 818 cases of cancer of the colon as a whole he found an average hemoglobin of 67.3%. In 4 cases reported by Clark et al (29) the hemoglobin averaged 47% and the red cell

mass 51% of normal. Brown et al (20) found 79% of their 60 cases of cancer of the colon had an anemia with less than 13 grams per cent of hemoglobin. Ten such cases are included in Schwertman's series (114) and had an average hemoglobin level of 10.8 grams per cent and red cell count of 3,900,000.

In 1927 Eisen (44) reported 19 cases of cancer in the pancreas and biliary system with an average hemoglobin level of 58% and a mean red cell count of 3,233,000. In 1928 (46) he reported 14 cases of biliary tract cancer with an average red cell count of 3,236,000 and hemoglobin level of 58%. Clark et al (29) reported 6 cases of pancreatic carcinoma in which the hemoglobin averaged 55% and the red cell mass 59% of normal.

In breast cancer the anemia reported is not as severe as that of the gastro-intestinal tract. Eisen (46) reports 52 cases in which the hemoglobin averaged 64% and the red cells 3,378,000. In the 130 cases of Alvarez et al (6) the average values were 74.4% and 4,410,000. In Morrison's (79) 8 cases the average hemoglobin was 69%. Ten cases reported by Schwertman (114) averaged 12.2 grams per cent hemoglobin and the mean red cell count was 4,300,000.

The degree of anemia in pulmonary cancer seems to be of about the same magnitude as that in breast cancer. Morrison (75) reports 15 cases with an average hemoglobin of 67%. An average of 74% hemoglobin with an average red cell count of 4,013,000 was found in 24 cases reported by

Eisen (46). Clark et al (29) found the red cell mass to be, on the average, 70% of normal and the hemoglobin 65% in ten cases of lung cancer.

In malignant disease of the kidney the anemia is also quite severe. Twelve cases of Eisen's (46) had an average hemoglobin of 64% and a mean red cell count of 3,730,000. In four cases reported by Schwertman (114), the average hemoglobin was found to be 11.7 grams per cent and the average red cell count to be 4,500,000.

Of the above writers, Schwertman (114) is the only one reporting cases of bladder cancer. He found in five such cases that the average hemoglobin was 11.7 grams per cent and the red cells averaged 4,110,000.

Commons and Strauss (31) reported three cases of prostatic carcinoma with associated skeletal metastases. The hemoglobin in these cases averaged 6.5 grams per cent and the red cells 2,400,000. Schwertman (114) reported ten cases and in these the values were 12.9 grams per cent and 4,390,000.

Schwertman (114) observed a mean hemoglobin of 11.1 grams per cent and red cell count of 4,001,000 in four cases of malignant disease of the ovary. Eisen (46) found the values to be 69% hemoglobin and 3,678,000 respectively in 13 cases included in his series.

Carcinoma of the uterus, cervix or body, is one of the commonest of malignancies seen, and here too, the associated anemia is quite severe. Eisen (46)

reported 15 cases with a mean red cell count of 3,175,000 and an average hemoglobin of 59%. Schwertman (114) in eight cases of cancer of the body found these values to be 4,150,000 and 11.5 grams per cent respectively. Alvarez et al (6) divided his cases into four groups. Fifty-five cases of cancer of the fundus without hemorrhage averaged 79.9% hemoglobin and 27 cases with hemorrhage averaged 61.8%. In 26 cases of cervical cancer without hemorrhage the hemoglobin average was 79.2% and in 29 cases with hemorrhage it was reduced to 64.5%.

3. Causes of Anemia in Cancer

a. Hemorrhage

Hemorrhage is one of the most obvious factors in the production of anemia in cancer. Cecil (27) and Schwertman (114) named this as one of the causes of this secondary anemia. Eisen (41, 42, 43) showed that the most severe anemia is associated with cases of malignancy where hemorrhage is a prominent feature, such as in lesions of the gastro-intestinal tract. Isaacs (70) attributed the anemia that occurs with malignancy to bleeding before any other cause. Shen and Homburger (115) attributed the anemia in 28.5% of their 193 cases to blood loss alone. Alvarsez et al (6) found the hemoglobin levels to be the lowest with those malignant tumors which had a large ulcerated area

from which blood could ooze. In other words, he judged that hemorrhage was a significant cause of anemia in patients with cancer and he supported this view by comparing cancer in the right and left colons. He found the anemia to be more profound with cancers of the right colon which tend to bleed more because of the larger area of their tumors as compared with those in the left colon. He also compared 55 cases of cancer of the fundus uteri with hemorrhage to 27 similar cases but without hemorrhage. These two groups had average hemoglobin levels of 74.9% and 61.8% respectively. In comparing 26 cases of cancer of the cervix uteri without hemorrhage to 20 such cases with hemorrhage the average hemoglobin levels were 79.2% and 64.5% respectively.

In contrast to the above, Ariel et al (9) could find very little correlation in their series between hypoproteinemia and anemia and the incidence of dietary intake or chronic hemorrhage.

Loss of blood could occur as chronic oozing from an ulcerated surface of a cancerous growth or it could take the form of an acute and perhaps massive hemorrhage as would occur in the erosion of an artery by a malignant process. But whichever form it takes, one would judge from the literature that it plays an important part in the production of this secondary anemia.

b. Bone marrow metastases

Cancers of the prostate, kidney, breast, lung and thyroid have a propensity for metastasizing to the bone marrow. This replacement of the bone marrow (usually the hemopoietic part, Rundles and Jonsson, (112)) has been given as one of the causes of anemia secondary to cancer. Isaacs (70), Haden (68), and Rundles and Jonsson(112) were of the opinion that bone marrow metastases play an important part in making the cancerous patient anemic. Vaughan (126,127), Thompson and Illyne (124), Mettier (78), Commons and Strauss (31) and Pisciotta (102) all described an anemia associated with bone marrow metastases in which the prominent features are a decrease in the number of red cells and the appearance in the peripheral blood of a large number of immature forms, both of erythroid and myeloid series. This anemia which they described is most frequently called, among other names, myelophthisic or leuco-erythroblastic anemia and is most commonly associated with skeletal metastases in carcinoma of the prostate.

In contrast to the above, Weisberger and Heinle (132) could find no correlation between the occurrence of anemia and the activity of the bone marrow. Morrison (79) found that the anemia in his series did not necessarily depend on metastases. Schwertman (114) states, "In some cases, even in the presence of metastases, anemia does not

occur." Shen and Homburger (115) concluded that anemia associated with cancer is not usually due to bone marrow replacement by neoplastic tissue. Vaughan (126, 128) observed that the anemia is not dependent upon mechanical blocking of the marrow but likens the anemia to pernicious anemia and suggests that it is caused by a deficiency of a factor or factors necessary for normal hemopoiesis.

Although there is much disagreement in the observations of the above writers the weight of opinion seems to warrant the view that, when they occur, metastases to bone marrow do in many cases contribute to the anemia secondary to cancer.

c. Dietary intake, digestion and absorption

Cancerous patients are often apathetic and as a consequence do not eat properly. In these patients, the possibility of anemia as a result of malnutrition must not be overlooked. Bateman (12) includes this factor in the causes of anemia in three of his patients. To this cause Eisen (45) also attributes some of the anemia seen in his patients.

Patients with malignant lesions of the oropharyngeal cavity or esophagus might suffer from anemia because of their discomfort in eating, and consequently an insufficient dietary intake.

Gastric achylia and obstruction in the gastro-intestinal tract are named by Eisen (44, 46) as two additional factors that must be considered. Gastric achylia would interfere with digestion, and obstruction would interfere with both digestion and absorption. However, Ariel (9) measured the nitrogen in the feces of 10 patients with gastric cancer and concluded that cancer did not interfere with digestion or absorption of proteins.

Totterman (125) found a low serum iron in a number of cancerous patients investigated and listed it as an etiological factor in the associated anemia. This low serum iron presumably results from an intake which is insufficient to meet the demands. Ariel (9) however, found that the long continued administration of large amounts of ferrous salts and liver concentrates did not significantly alter the blood picture in patients with gastric cancer.

d. Hepatic insufficiency and defective protein metabolism

An adequately functioning liver is necessary for the fabrication of proteins used in the synthesis of hemoglobin. Therefore, hepatic insufficiency resulting from secondary growths in the liver or its depression as

The first thing I noticed when I stepped
out of the car was a warm, humid breeze.
It felt like a giant hand reaching out to
greet me. The air was thick with the scent
of tropical flowers and the distant call of
birds. I took a deep breath, savoring the
moment. The sun was shining brightly,
casting a golden glow over everything.
I felt like I had entered a new world.

As I walked along the path, I noticed
how the light filtered through the trees.
It created a magical atmosphere, with
dappled sunlight hitting the ground.
The path was well-maintained, and the
scenery was breathtaking. I saw a small
stream flowing gently, its surface reflecting
the surrounding greenery. The water was
clear and cool, and I could hear the
soft rustle of leaves as the wind blew.

I continued to walk, feeling more at
ease with each step. The landscape was
diverse, with a mix of lush vegetation
and open fields. In the distance, I saw
rolling hills under a vast, blue sky.
The air was fresh and invigorating, and
I felt a sense of peace and tranquility.
It was a beautiful day, and I was
grateful for the experience.

a whole by toxic substances possibly produced by malignant cells could cause anemia. This factor has been mentioned by Oppenheim (87) in his series of cases of carcinoma of the gastro-intestinal tract. However, it must be remembered that the liver has a great reserve and metastases in it have to be extensive before any insufficiency is manifested. Ariel (9) suggested that the hypoproteinemia and anemia in gastric cancer were caused by defective fabrication of proteins.

e. Hemolysis

Intravascular hemolysis as a result of the release of a hemolysin by malignant cells has been postulated as another mechanism in the production of anemia secondary to cancer. Stats et al (120) note that metastatic carcinoma can cause a hemolytic anemia and Isaacs (70) speaks of a hemolytic anemia classically found in cancer of the stomach and liver but doesn't report any cases. Shen and Homberger (115) attribute to hemolysis the anemia seen in 2.6% of their 193 cases of cancer in various organs. Waugh (131) reports 2 cases of hemolytic anemia associated with carcinoma. Singer and Dameshek (116) list carcinoma as a cause of hemolytic anemia and report one case of dermoid cyst of the ovary. Allibone and Collins (5) report one case in which the

hemolytic process was arrested following excision of a cystic teratoma of the right ovary. (Note: The spleen was not excised.) However, in this case they could not demonstrate any hemolysins or atypical agglutinins.

Eisen (46) judged hemolysis as not being significant in the cases he reported.

The above instances do not make up a large proportion of the malignancies encountered and it would seem that hemolysis is of little significance in the majority of malignant diseases.

f. Intoxication from necrosis and infection of malignant neoplasms

There is the possibility that degeneration or toxic products from necrotic or infected parts of neoplasms could cause anemia by a toxic effect on the liver (with the resultant defective fabrication of proteins as mentioned previously) or on erythropoietic tissue, as well as being the cause of intravascular hemolysis. Eisen (44, 46) observed that adenocarcinoma with its greater tendency to ulceration and infection was associated with a greater degree of anemia than scirrhus, colloid or simple carcinoma. Schwertman (114) too, noticed that anemia is more marked in cancer

of those organs which are more susceptible to ulceration and necrosis. Totterman (125) included secondary infection as one of the causes of anemia in cancer. In speaking of carcinomas of the colon, Alvarez et al (6) found a definite relation between the size of tumors removed at operation and the degree of anemia. Thus he concluded, "The essential factor in the production of the anemia seems to be in the presence of a large ulcerated area from which blood can ooze and through which bacteria can enter." Evidently, he was of the opinion that secondary infection contributed to the production of the anemia.

g. Specific erythropoietic inhibitor produced by malignant cells

An anemia frequently exists in conjunction with malignant disease which cannot be explained on the basis of any of the above causes. Eisen (46) makes the statement that sometimes the anemia seen in patients with malignant disease is out of proportion to the actual lesion. Totterman (126) makes mention of an anemia "caused by the malignant neoplasm as such - true cancer anemia." Many of the 130 cases of non-ulcerating cancer of the breast reported by Alvarez et al (6) would perhaps fall into this group as well as some of his cases of cancer of the fundus uteri and cervix uteri uncomplicated by hemorrhage.

Clark et al (29) speak of a fundamental disturbance in hemoglobin metabolism in malignancy. They also refer to a statement of Krache's in which he suggests that the malignant process either elaborates a product capable of bone marrow inhibition or utilizes substances necessary for red cell production. Haden (65, 66) cites two case reports of gastric carcinoma in which he explains the associated anemia on the basis of a depression of the bone marrow in one case and of toxic interference with the synthesis of hemoglobin in the other. Morrison (79), Schwertman (114), and Shen and Homberger (115) have all postulated the production of a toxic substance by malignant cells which depresses the bone marrow. Morrison calls it a carcinomatoxin. Such a substance by inhibiting the bone marrow could explain the anemia seen in cancerous patients which cannot be accounted for by any of the above causes.

B. Anemia and Lowered Liver Catalase in Experimental Animals with Tumors.

1. Occurrence of Anemia

Paralleling the occurrence of anemia in patients with cancer, workers with experimental animals have noticed the occurrence of anemia in those with tumors. It is realized that caution must be exercised

in the interpretation of results in experimental animals and in its application to humans, but it is felt that the results in experimental animals in this respect closely parallel the same phenomenon in humans and therefore should be noted.

In a review article in 1943, Greenstein (62) noted that a decrease in the hemoglobin level was one of the marked systemic effects in animals of tumors in general. Dickinson and Begg (34) observed a significant degree of anemia in rats with benign tumors. They also observed (14) anemia in force-fed rats bearing tumors. An anemia in mice and rats bearing tumors was reported by Blumenthal (19). However, he noticed that the anemia did not become pronounced until the tumors were large and the animals were markedly cachectic. Pearce and Casey (99) found a decreased red cell count and a lowered hemoglobin in rabbits when the tumor process became pronounced. In working with embryonic chicks inoculated with mammary mice carcinomas, Taylor, McAfee and Taylor (123) reported that hemoglobin levels were depressed by as much as 70% by the eighteenth day.

Taylor and Pollack (122) administered methylcholanthrene to mice and butter yellow to rats. They found that the hemoglobin was depressed in the resulting precancerous conditions and that it became more

marked when tumors appeared. Somewhat related to this are the findings of Strong (121). He reported an early precocious drop in the hemoglobin level in cancer-susceptible strains of mice but clearly differentiates it from the anemia secondary to malignancy. Davis too (33), reported a lower hemoglobin, red cell count and red cell volume in tumor strain mice as compared to non-tumor strain mice.

2. Mechanism of Production of the Anemia

Cliffton and Wolstenholme (30), Sobel and Furth (119), Furth and Sobel (54), and Furth and Moshman (55) noted an overall increase in blood volume in their tumor-bearing mice. If the plasma volume increased more than the cell volume the anemia described above by various workers might only be apparent instead of real.

On the other hand, Taylor, McAfee and Taylor (123) suggested that a substance might be produced by the tumor cells and when released into the blood stream would have some inhibiting influence on the concentration of hemoglobin in the blood. Armstrong and Ham (11) thought that the anemia may be due to some agent associated with the tumor rather than the production of some substance by the tumor cells themselves.

Both of these last two postulates parallel closely the theory of an erythropoietic inhibitor produced by malignant cells described above in relation to anemia in cancerous patients and serve to reinforce it.

3. Association of Anemia and Lowered Liver Catalase Activity in Tumor-Bearing Animals

Greenstein (62) noticed that the two most marked systemic reactions in tumor-bearing animals are a decrease in liver catalase activity and a decrease in the hemoglobin. These two substances are both conjugated proteins, and the hematoporphyrin prosthetic group is common to both. On the basis of this fact Greenstein reasoned that an interference with the synthesis of the hematoporphyrin group by some mechanism could explain both the anemia and the lowered liver catalase activity. Appleman et al (7, 8), Dounce and Shanewise (36), Begg (13), and Greenstein et al (61) are among those who have observed this lowered liver catalase activity in tumor-bearing animals (rats). Begg and Dickinson (14) noticed that forced-feeding maintained the carcass weight but the loss of liver catalase activity was still present in the tumor-bearing rats. Dickinson and Begg (34) noticed a more pronounced fall of liver catalase activity with malignant tumors than with those which were benign. Adams (1) found that the injection of coarse homogenates of certain sarcomas depressed the liver catalase activity within 48 hours and maintained this depression for about two days. He also noted that injection of disintegrated material still had an effect even in the absence of observable tumor growth. Greenfield and Meister (60)

observed a depression in the liver catalase activity by more than 50% within 24 hours following intra-peritoneal injection of ethanol fractions of C3HBA mammary tumor. They also found (59) that extracts of human cancers had the same effect.

In contrast to the above, Klatt and Taylor (72), and Skavinski and Stein (117) found the total liver catalase activity to be unaffected or even higher in tumor-bearing mice (Klatt and Taylor) and in chick embryos with tumor implants (Skavinski and Stein).

In order to explain the lowered liver catalase activity which they observed, Greenstein et al (61), Greenfield and Meister (60), and Adams (2) have postulated the elaboration by the tumor of some toxic substance which acts directly or indirectly on the liver to produce the above effect. Appleman et al (8) suggested that there may be a competition between the liver and the tumor for some substance necessary in the formation of the catalase.

This association between anemia and lowered liver catalase activity in tumor-bearing animals is evidently more than coincidental and it would seem reasonable to agree with Greenstein (62) that the tumor has some systemic effect on the animal which interferes with the synthesis of the hematoporphyrin group.

C. Review of Blood and Bone Marrow Culture Methods

1. Cultures in Solid Media

Downey (37) reported that Carrel and Burrows were the first to culture bone marrow. In 1910 they grew explants of bone marrow from the femur of a young cat, presumably in a plasma clot. Three years later Foot (53) became the next worker who successfully cultured bone marrow. He teased out bits of chicken bone marrow in Locke's solution which he then placed in drops of plasma on sterile cover-slips. When the drops had clotted he inverted the cover-slips on hollow ground slides and sealed them with vaseline. During incubation he observed that different cells migrated out from the bone marrow particle and began multiplying in the surrounding medium. After a desired period of incubation the cultures could be examined microscopically by the usual histological techniques of fixing, sectioning and staining. Erdmann in 1917 (50) followed closely the method used by Foot and also employed chicken bone marrow. After various periods of incubation she removed the original explant of bone marrow and introduced it into a new drop of plasma. By making similar transfers at various intervals of time after the start of incubation she was able to study migrations of different cell forms from the same bone marrow particle until it was exhausted. Carrel and Ebeling in 1922 (26) reported observations on cultures of the coagulated buffy coat (leucocytes) of centrifuged

peripheral blood from chickens. Using the above method they studied the effects of such media as plasma, plasma and Tyrode's solution, plasma and chick embryo juice and Tyrode's solution. Lewis (74) employed cultures of whole avian and mammalian blood in the above manner in 1925 to determine the changes which took place in the leucocytes and in 1926 Lewis and Lewis (75) studied the transformation of mononuclear blood-cells from lower vertebrates in hanging-drop cultures. This method was also used by Pierce in 1932 (100) in studying the buffy coat in leukemic blood. In his cultures he employed a medium of Tyrode's solution, rabbit plasma and rabbit embryonic extract. In 1943 Rachmilewitz and Rosin (110, 111) placed particles of bone marrow in test tubes in which there was a medium consisting of plasma, Tyrode's solution and chick embryo juice. They closed the test tubes, and after a variable period of incubation, examined the cultures by the usual histological methods.

Armbrust and Bett in 1946 (10) obtained satisfactory growth of bone marrow explants from guinea pigs using a medium of 3% agar, Tyrode's solution and sheep's plasma. Pieces of bone marrow were planted in this medium just as it was beginning to coagulate. Cells migrated from the explants out into the medium and growth was measured by determining the increase in area of each explant.

2. Cultures "in Vivo."

Two methods of blood and bone marrow culture "in vivo" have been described. The first is that of Pierce (101) in which he planted particles of the coagulated buffy coat of centrifuged leukemic blood on the exposed chorioallantoic membrane of chicken embryos. After the 'plant' had been made the window in the eggshell was covered with a cover-slip and sealed with paraffin. The eggs were incubated again thus permitting the growth of the leucocytes for a short time. Plum in 1950 (107) described the second method of culture "in vivo." In this method a suspension of bone marrow cells is injected into the anterior chamber of the eye of the white rat. After the desired length of time the contents of the anterior chamber are aspirated, smeared, stained and examined. Plum found this method to be advantageous in studying the differentiation of cells.

3. Culture of Blood or Bone Marrow Cells in Suspension

In 1936 Osgood and Muscovitz (88) devised an apparatus in which a liquid suspension of bone marrow cells was cultured. Osgood made this comment in describing his apparatus. "The apparatus supplies a lung, kidney, and circulation for the marrow, as it provides for the control of oxygen and carbon dioxide tension, of pH, and of the composition of the medium; for elimination of waste products, for supply of nutrients, for removal of

part or all of the culture for study, and for maintenance of sterility."

The essential feature of this apparatus was the semipermeable membrane in the form of a bag (parlodion) which contained the bone marrow and separated it from the surrounding medium. This medium outside of the parlodion bag containing the bone marrow was circulated continually, thus keeping the environment of the bone marrow suspension constant. Nutrient materials could diffuse through the membrane into the culture and as waste products accumulated, they could diffuse out of the culture and be carried away by the circulating medium. Gas of known composition was bubbled through the bone marrow suspension by means of a tube leading into the parlodion bag. This gas served both to aerate the culture and to keep the cells in the suspension from settling out. The part of the apparatus which contained the bone marrow was incubated at 37.5° C.

Using this apparatus Osgood and Muscovitz were the first to culture human bone marrow. Bone marrow obtained by sternal puncture was diluted with a diluting fluid and then introduced into the parlodion bag along with some nutrient medium such as serum. Cell counts were performed on, and smears made of the suspension at this stage to be compared with those made after a certain period of incubation at 37.5° C. Certain other determinations such as hemoglobin estimation and chemical analyses were also done at various intervals.

The above apparatus was expensive, difficult to build, and sterility was sometimes hard to maintain. Therefore, after a few months Osgood abandoned this method in favor of a simpler one (Osgood and Brownlee; 89, 90). In his second method 30 cc. vaccine vials, with rubber caps were used as the culture flasks. Media and cells were transferred using sterile syringes and needles.

Marrow obtained by sternal puncture was placed immediately into a vial containing a sterile citrated physiological salt solution. After centrifuging this vial, the immature cells could be largely separated off from the red cells. These immature cells were then transferred to other vaccine vials and diluted to a desired concentration with a physiological salt solution and placental cord serum in the ratio of about 2 : 1. Cell counts and smears were made at this time and then the vials were incubated at 37.5° C. Serial counts and smears were made after various periods of incubation. About every forty-eight hours the medium in the vials was changed by centrifuging the vials and replacing the supernatant fluid with an equal quantity of fresh medium. The air in the vials was replaced about every four days.

Using this method Osgood found that he could incubate a large number of cultures at one time with a minimum of work and expense. However, finding it desirable culture larger quantities of bone marrow at once, he (93) devised a third method in 1938. He claimed that this

third method had all the advantages of his first method which had been partially sacrificed in the method employing vaccine vials, and yet was much simpler than the method originally devised.

The bone marrow suspension in this method was contained in a large pyrex aspirator bottle. Fresh aerated fluid medium flowed into the culture bottle from the bottom and at the same time an equal volume of used medium was withdrawn from the top of the fluid level in the flask. In other words, new medium flowed in at the bottom and upwards through the suspension of cells to the upper levels of the fluid where it was withdrawn. Because the cells settled to the lower parts of the fluid in the culture bottle no cells were lost with the outgoing medium. When a sample of the culture was desired for various determinations, the culture flask was shaken in order to suspend evenly the cells in the fluid and thus to insure the withdrawal of a representative sample.

In evaluating the above three methods of bone marrow culture which Osgood and his co-workers have devised it is of significance that in later work (22, 91, 92, 94, 95, 96) which was carried out on a number of problems the second method, that which employed vaccine vials, was favored.

In 1946 Plum (103, 105) began culturing bone marrow in order to study, amongst other problems, reticulocyte ripening substances and erythropoiesis in general. He

judged that the method of Osgood and Muscovitz (88) was the most physiological method that had been devised for the culture of bone marrow up to that time. He therefore built an apparatus similar to that described by Osgood and Muscovitz and used it with good success in culturing bone marrow cells. However, since this apparatus did not allow direct microscopic examination of the cells while they were in culture, he developed a method of his own (103, 105) in which this was possible and used it in conjunction with the above in his observations of erythropoiesis.

In Plum's microscopic method (103, 105) a small quantity of bone marrow cells in suspension was contained in a small hole in a cover-glass, bounded on top by another cover-glass and underneath by a colloidion membrane. The colloidion membrane was supported underneath by another cover-glass similar to the one containing the bone marrow suspension so that the hole in the lower cover-glass was directly under that of the one above. The lower cover-glass was cemented to a Bürker-Türch counting chamber, through the gutters of which medium flowed in a continuous stream. In principle this method was the same as the macro method (Osgood and Muscovitz) in that the bone marrow suspension was separated by a semi-permeable membrane from a nutrient medium which was being replaced constantly. This whole apparatus could be placed under

a microscope thus allowing the cells to be viewed directly while they were in culture. A heating table under the microscope kept the temperature at the desired level. Plum found that the phase contrast microscope facilitated the observations which he made using this method. In addition to the above, Plum (107) also devised an "in vivo" method which has been described previously. Using the above methods, Plum (106) has successfully cultured bone marrow from rabbits, dogs, cats, and humans.

A comparatively large number of workers has cultured bone marrow and blood using the method of Osgood and Brownlee or modifications thereof and found the method applicable to a number of research problems. In 1940 Israëls (71) found the method suitable for studying the development and morphologic changes of leucocytes from human bone marrow in culture. Hays (69) and Gunz (63, 64) have successfully used the method in the culture of peripheral blood cells. Hays in 1946 studied the effect of folic acid on red blood cells and in 1948 Gunz studied human leukemic cells in culture. In 1947 Rusznyak et al (113) studied the maturation of megaloblasts in cultures of bone marrow from patients with pernicious anemia. Norris and Majnarich (80, 81, 82, 83, 84, 85, 86), and Bieselle and Berger (17) tested the effects of various substances on bone marrow cultures. They modified Osgood's and Brownlee's technique by placing glass beads in the vials and then placing the

vials in a rack in a Warburg bath so that the cultures were slowly agitated while they were being incubated. Norris and Majnarich (80) were able to culture bone marrow of cattle, rabbits, sheep, rats and cats using this modification of Osgood's and Brownlee's method.

SECTION III

SURVEY OF PRESENT CONCEPTS OF ERYTHROPOIESIS

As this thesis deals largely with bone marrow cultures and especially the erythropoiesis in these cultures, it would seem imperative that a discussion of erythropoiesis in general should be included. However, it must be remembered that this project does not deal with erythropoiesis per se, but rather with the establishment of a biological assay method which may make the confirmation of the presence of an erythropoietic inhibitor in malignancy possible. Nevertheless, it is felt that knowledge of erythropoiesis is needed as a background to the work.

When this project was begun it was generally accepted that red blood cells were produced in the bone marrow from the normoblasts found there. However, papers by two authors who disagree with this concept have been noted. In 1943 Duran-Jorda (38) put forth a theory of erythropoiesis deviating widely from any proposed up to that time. He regarded the red blood corpuscle as a product of cellular secretion and not as one of cellular evolution. In examination of embryo slides he noticed certain cells with cartlike nucleii whose cytoplasm contained bodies which appeared to be red blood corpuscles. He found that some of these cells had split into two portions. The one part contained the nucleus and looked like a plasma cell. The other part consisted of some of the spheroidal bodies which had become free. From these observations he concluded that the red blood corpuscle was a product of a unicellular gland system. In this system the plasma cell develops red blood corpuscles in its cytoplasm. When the cell reaches a certain size it breaks in two and thus liberates the red blood corpuscles. The part of the cell containing the nucleus looks like an erythroblast at this point. In its subsequent development Duran-Jorda visualized it as becoming a normoblast, then a lymphocyte, and finally a plasma cell, thus completing its cycle and back to the stage once again where it could produce red blood corpuscles.

In two later papers (39, 40) he had modified the above conclusions. He suggested that the lymphocyte through the intermediate stage of the Paneth cell becomes the eosinophil cell which he found in the gastric and intestinal mucosae. These eosinophil polymorphs gained entrance to the blood and were carried to the hemopoietic organs. He looked upon the granules in the cytoplasm of the eosinophils as preformed red corpuscles which increased in size and with the rupture of the eosinophil were released into the circulation. The eosinophil then became a lymphocyte once more and could therefore repeat the above process. In 1950 Duran-Jorda (41) reported that red blood corpuscles in the embryo were a product of normoblastic extrusion and that in the adult the normoblast secretes the reticulocyte in the bone marrow (cf. Plum, 108).

In 1947 Wajda (129, 130) proposed another concept of erythropoiesis. He made observations of the changes that take place in traumatized striated muscle fibers and reported that he saw red blood corpuscles arise from the degenerating muscle fibers. He supported his views with published photomicrographs which lend the support of direct observations. However, Le Gros Clark (73) challenged the above theory and stated that he was familiar with the changes in traumatized striated muscle and could not find any process even remotely similar to those observed by Wajda.

The above concepts are indeed startling and seem to be incompatible with the classical theory of red blood cell formation in the bone marrow from the cells of the reticulo-endothelial system. As this thesis does not deal primarily with erythropoiesis, no studies were made in order to confirm or disprove the above theories of red blood cell formation. Instead, the usual theory of erythropoiesis has been accepted and used as a basis on which to work.

Doan et al (35) attribute to Neumann the first description of erythrogenesis in bone marrow which he made in 1868. Since then numerous workers have confirmed the normal production of red blood cells in adult life by the bone marrow. Cells from the reticulo-endothelial system (which arises from the mesodermal layer in the embryo) are the precursors of red blood cells. The first recognizable cell of the erythroid series of cells in the bone marrow is the pronormoblast (following the terminology which Wintrobe used in his textbook on hematology). This is a fairly large cell with a large, somewhat vesicular nucleus containing one or more nucleoli. Surrounding the nucleus is a thin band of basophilic cytoplasm. In the course of the cell's development the nucleoli disappear and the chromatin in the nucleus takes on a coarser appearance, and may even arrange itself in a manner similar to that of the chromatin in a plasma cell. At this stage

the cell is called a basophilic normoblast. With the appearance of traces of hemoglobin in the cytoplasm, the cytoplasm takes on a polychromatic character and the cell is known as a polychromatic normoblast. The nucleus as well as the cell as a whole is smaller and the chromatin is more 'clumped' by this time. This cell becomes the orthochromatic normoblast when the cytoplasm becomes almost completely acidophilic, indicating that the cell has acquired almost its full complement of hemoglobin. At this point the nucleus becomes more and more compact until it finally becomes pycnotic and is lost. With the loss of the nucleus the cell becomes the adult red blood cell and is ready for its release into the circulatory system. Some remnants of the basophilic network of the cytoplasm of the early stages (such as the pronormoblast) may remain for a short while. As long as there is any of this basophilic material left in the cytoplasm the red blood cell is known as a reticulocyte.

All the cells in the above series up to the orthochromatic normoblast with the pycnotic nucleus are able to divide mitotically. Once the nucleus becomes pycnotic, however, the power of multiplication by mitosis is lost.

There has been much controversy in the literature concerning the mechanism by which the nucleus of the normoblast is lost. The two most prevalent concepts of the fate of this nucleus are:

(1) That it dissolves or is broken up inside the orthochromatic normoblast by the process of karyolysis.

(2) That it is extruded from the cell and is subsequently broken up outside of the cell.

There is another theory as to the mechanism of production of erythrocytes from normoblasts, but which has received relatively little attention, and yet which seems plausible enough to receive serious consideration. In 1912 Emmel (47) made observations on the blood of pig-embryos which convinced him that normoblasts give rise to erythrocytes by a process of budding. In 1914 (48) he reported more evidence for this concept and in 1924 (49) after further studies of the blood of amphibia as well as of embryos of mammals, he was convinced that erythrocytes did arise by budding or cytoplasmic segregation from the normoblasts. Plum (103, 106, 108) studied this phenomenon using his micro-culture apparatus. He reported that he observed a spherical erythroblast assume an oval shape with the nucleus at one end and the bulk of the cytoplasm containing hemoglobin at the other end. After a short time a portion of this cytoplasm would become detached and in about an hour become a cell of normal size. In his macro apparatus he noted that the addition of colchicine which completely inhibits mitosis had hardly any effect on the rate of red cell production. Also, on a theoretical basis he calculated that there were not enough mitotic figures relative to the total number of normoblasts

in the bone marrow to produce the required number of erythrocytes per day. From the above studies Plum concluded that erythrocytes are the product of cellular secretion by gemmation or budding and not of cellular evolution.

SECTION IV

CRITIQUE OF METHODS OF ERYTHROPOIETIC STUDIES

A. "In Vivo" Methods Using Experimental Animals

Presuming that an erythropoietic inhibitor is released into the blood stream by malignant cells, some means of its extraction from the blood and injection into experimental animals could be reasonably expected. If this substance was excreted by the kidneys of cancerous patients it may also be found in and extracted from the urine. The injection into experimental animals of such extracts might be expected to lead to anemia in them.

Certain difficulties must be considered in the evaluation of the use of experimental animals in such a project. Firstly, a sufficiently large number of animals would have to be used in order that various extracts from a number of patients with cancer could be tested. Secondly, the best site of injection would have to be determined by comparing the results of tests on injections made intra-venously, intra-muscularly, intra-peritoneally, and sub-cutaneously. Thirdly, the extracts would have to be of such a nature that various injection reactions such as abscesses and allergic reactions would not result. Fourthly, and perhaps most important, would be the time lapse which may result before any anemia in these animals would show up after an injection. If the above reaction could be used in the diagnosis of malignant disease this time lapse may be of sufficient magnitude to markedly reduce the value of the reaction for clinical diagnosis of malignancy. For these as well as other reasons it was decided that the intact experimental animal might not be satisfactory biological material for the determination of an erythropoietic inhibitor in malignancy, keeping in mind the possible clinical value of a quick method.

B. "In Vitro" Methods (Bone Marrow Cultures)

An erythropoietic inhibitor should logically be expected to exert its effect "in vitro" as well as

"in vivo." In other words if it produces an anemia in cancerous patients by inhibiting the bone marrow it should by the same action depress the growth and multiplication of bone marrow cells in culture. Since the growth in these cultures can be determined at frequent intervals, any effect of extracts of blood on these cultures should be quickly determined.

Of the many methods of marrow culture which have been developed, some are superior to others. Therefore it was expedient that each method be analyzed in the light of the problem being studied (anemia in cancerous persons) in order that the method most suited to the demands of the project be chosen and employed. These demands are as follows:

(a) Simplicity. This would facilitate the adoption of the method as a whole should it become practical to use it as a diagnostic measure.

(b) Optimal physiological conditions, in order to obtain the maximum growth and multiplication possible.

(c) Rapid growth, which would give a greater sensitivity in detecting the presence of an inhibitor.

(d) Ease and accuracy in determining growth.

(e) Feasibility of incubation of multiple cultures so that there would be proper controls for each experiment, and so that many tests might be run simultaneously.

The bone marrow culture methods which have been included in the review of the literature will be compared under a number of headings in the light of the above criteria.

1. Assessment of Growth

Any of the methods employing bone marrow clots in solid media have the disadvantage that it is extremely difficult to assess accurately their growth. Multiplication is usually determined by noting the increase in area of the explanted bone marrow particles as did Armburst and Bett (10). It is obvious that such a method might be inaccurate because it does not consider any increase in thickness or in the concentration of cells in a given area. Enumeration of the nuclei or cells in such cultures is hardly feasible because of the thickness of the clot and the consequent superimposition of the nuclei.

Cultures of bone marrow cells in fluid suspension seem more amenable to accurate assessment of their growth. The production of red blood cells can be estimated from changes in their concentration from time to time. Three methods of calculating red cell counts are reviewed by Connolly in 1947 (32). The oldest and most widely known and used method is that employing

a hemocytometer. The second that he reports is that of Phillips in which the number of red cells is estimated from the grams of hemoglobin as determined by their copper sulfate specific gravity method. The third method and the one which Connolly recommends is the photo-electric method introduced by Blum (18) in which the red cell count is estimated from the turbidity of a diluted solution of red cells as measured by a photo-electric colorimeter. Increases in the number of nucleated cells can be estimated by determination of uptake rates of radioactive phosphorus in desoxyribose nucleic acid (Osgood, 97) or by repeated nucleated cell counts with a hemocytometer. Direct counts of cells in a hemocytometer is the only method of the above applicable to both red and nucleated cells and therefore is perhaps the most suitable method of estimating cell counts in fluid bone marrow cultures. Accordingly it was decided to use the standard methods of determining cell counts in this project and a discussion of the technique of such will be given later.

Assessment of growth in the micro method of Plum would seem to be almost impossible. The sample is too small to allow any determinations such as cell counts by the usual methods or by colorimetric determinations to be made. Even direct enumeration of the number of cells in the 'culture chamber' is impossible because there is a certain amount of the initial sample that is squeezed out

between the two upper cover-glasses and between the cover-glass with the bone marrow suspension and the colloidal membrane. There is a constant shifting of cells from the 'hole' containing the bone marrow out to the space between the cover-glasses and back again. Such 'migration' of these cells seriously handicaps an evaluation of the concentration of cells and consequently of the increase in the number of cells.

The two "in vivo" culture methods of Pierce (101) and Plum (107) are included in this discussion of "in vitro" culture methods simply because they are means of artificially growing bone marrow cells. The same difficulties in assessing growth are found in the Pierce method of culturing bits of bone marrow (or clotted leucocytes) on the chorioallantoic membrane of the chick embryo as are found in all the methods of culturing bone marrow in solid media as discussed above. In the Plum method in which suspensions of cells are cultured in the anterior chamber of the rat's eye it is difficult to conceive a technique whereby one could withdraw a representative sample of the cell suspension. One could hardly insure an even suspension of cells in the anterior chamber by shaking. These two methods of bone marrow culture would be of value in studying the morphology of cells rather than their rate of multiplication.

2. Cell Counting Technique

Before the technique of culturing bone marrow cells in suspension was begun the method of cell counting using a standard hemocytometer was chosen for determining the multiplication of cells in these cultures. The method is a modification of that used in making routine clinical red cell counts. The technique of making red cell counts will be reviewed first.

The materials used for red cell counts are as follows: a lancet, a blood diluting pipette, a mechanical shaker, a hemocytometer, a microscope and a tally device. The lancet is used for making a small skin puncture to obtain a sample of capillary blood. The pipette allows the blood to be diluted 1:1000, 1:500, 1:333.3, 1:250, 1:200, 1:166.6, 1:142.8, 1:125, 1:111.1 or 1:100. One in two hundred is the dilution normally employed but 1:100 is recommended for making counts on blood in which the red cell concentration is markedly reduced. The mechanical shaker is not essential as the pipettes can be shaken by hand, but it insures a more even distribution of cells in the pipette and does the job more quickly and easily. The hemocytometer is a heavy glass slide with three shoulders running across it. The outer two shoulders support a glass coverslip 1/10 millimeter above the center shoulder. This center shoulder is divided into two parts by a trough running

parallel to the length of the slide. Each half of the center shoulder has a small grid inscribed on it. This grid is a square of 9 square millimeters in which the center square is divided into 25 small squares each of which is further divided into 16 more squares. The four square millimeters in the corners of the large square are divided into 16 squares each. The cells from a diluted sample of blood are counted in this hemocytometer under a microscope. A hand tally greatly facilitates the counting of the cells.

A red cell count is done in this manner. Blood from a small puncture is drawn up to the 0.5 mark on the blood diluting pipette. Diluting fluid such as Hayem's, Gower's or Toisson's is drawn up to the 101 mark thus giving a dilution of 1:200. The pipette is shaken by hand for about three minutes or on a mechanical shaker for about thirty seconds. Three or four drops are then shaken out and discarded before the counting chamber or hemocytometer is filled or charged with a drop of diluted blood on each side. After the cells have been allowed to settle in the hemocytometer for about a minute the cells in five of the $1/25$ of a square millimeter squares in the center square are counted. These five squares are the four corner squares and the center one of the larger central square millimeter. In other words, the number of cells in 80 of the smaller squares in the central square millimeter are counted. This number multiplied by the dilution factor (200), the

area factor (which is 5 because the cells over $5/25$ ths of a square millimeter were counted), and the volume factor (which is 10 because the chamber is only $1/10$ of a millimeter deep) gives the number of red blood cells in one cubic millimeter.

In making white blood cell counts the same procedure is followed in principle except that only a 1:20 dilution is made, the diluting fluid (such as 1% acetic acid) used dissolves the red blood cells and the cells in the four corner square millimeters are counted. Because of the different dilution and different area involved the number of cells counted is multiplied by a different factor.

Cell counts as performed by the standard method are liable to the following errors (21).

(a) Sampling errors of which there are two; the first in taking the sample of blood for dilution and the second when the diluted blood is placed in the counting chamber.

(b) Dilution error which can arise from an inaccurately calibrated pipette or from lack of experience on the part of the technician.

(c) The chamber error of Berkson, Magath and Hurn (15, 16). This error consists of three "sub-errors."

- i. Inconsistent filling of the counting chamber.
- ii. Random settling and distribution of cells within the chamber.

iii. Variations in dimensions of the chamber that arise from inaccurate calibration or improper assembly of the cover-glass and chamber after cleaning.

(d) Actual errors in counting.

Some of these errors are inherent in the technique but others are subject to a human factor, and will depend in some degree at least on skill of performance. A clean puncture wound from which the blood flows freely will insure a more representative sample than one from which the blood has to be squeezed. Only pipettes and counting chambers approved by the United States Bureau of Standards should be used. Using these pipettes and after sufficient practice a high degree of skill can be attained in drawing the blood to the 'mark' accurately and in making an accurate dilution of this blood by drawing diluting fluid to the second mark (the 101 mark which is situated above the bulb of such a pipette). With respect to diluting fluids Ying-Chang Ch'u and Forkner (134) found Gower's solution to be superior to Hayem's solution which often clumped the cells. The shaking of the pipette before the chamber is charged is very important. This is done best with a mechanical shaker specially designed for the job. With practice, however, one can learn the technique of shaking these pipettes by hand. The pipette must be shaken in a plane at right angles to the long axis of the pipette and for a period of about three minutes if

it is done by hand or about thirty seconds if a mechanical shaker is used. Immediately on completion of the shaking about three or four drops must be discarded from the pipette to avoid filling the chamber with fluid that was in the capillary part of the pipette and which is therefore not a representative sample of the diluted blood. As the cells settle fairly rapidly after the shaking is completed only one side of the counting chamber should be charged before the pipette is shaken again and the second side charged. In charging (or filling) the chamber care must be taken neither to over-charge nor under-charge it as this may effect the distribution of the cells. Sufficient time must elapse before counting is started so that the cells can settle to the surface of the chamber before they are counted. In this way cells will not be missed through being out of focus. The random settling of the cells in the chamber is one factor largely out of control of the technician but a clean chamber and smooth, even filling of the chamber help to give an even distribution. Errors in the actual counting of the cells can be reduced or eliminated by care and practice. In the cleaning and reassembly of the counting chamber special precautions must be taken to clean off any lint or dirt which may be on the shoulders which support the cover-glass as such foreign material between the cover-glass and the shoulders would materially affect the depth of the chamber. Care should also be taken to orientate the cover-glass on the

chamber each time so that if there is a bow in the cover-glass for example it will always be either up or down. Clips (133) which hold the cover-glass in place have been found to decrease the variations in the dimensions in the depth of the chamber from one assembly to the next.

3. Sterility

Bone marrow cultures provide ideal conditions for the growth of many bacteria. The temperature is optimal for many of them and there is an excellent medium in which they can grow. Their presence is obviously undesirable and precautions have to be taken to keep the cultures free of contamination, or at least to keep the number of bacteria as low as possible.

All equipment which comes in contact with the actual culture material has to be sterilized by autoclaving, dry heat, boiling or by chemical means. Consequently, a method of bone marrow culture in which the amount of equipment used is kept to a minimum will entail a proportionately smaller amount of time and work in the preparation of equipment for the experiments. The apparatus which Osgood (88) first designed was complicated and cumbersome and therefore difficult to sterilize. This was perhaps one of his reasons for the abandonment of this method in a few months in favor of a simpler one in spite of the fact that this was one of the best marrow

culture methods yet devised from the standpoint of physiological conditions. This second method which employed vaccine vials required less work in proportion to the number of cultures incubated in that there was less equipment to clean and sterilize. For the purposes of this project a method such as that of Osgood and Brownlee (89, 90) in which the amount of equipment used is kept to a minimum is desirable in view of the possible practical application of the technique in the diagnosis of malignancy.

Aseptic technique is of paramount importance in the maintenance of sterility in these cultures. Draft-free rooms, a minimum of activity while working with the cultures, care in keeping the culture rooms as dust-free as possible, flaming the mouths of culture flasks whenever they are opened, and bacteriological technique in general will greatly reduce the chances of contamination. However, in the event that a few bacteria do gain entrance to the cultures and the circumstances there are favorable for their growth, a certain interval of time is required before they can establish themselves and multiply sufficiently in number so that they interfere with the growth of the bone marrow cells. If this period of time in which the bacteria are establishing themselves (and in which they, presumably, are not interfering with erythropoiesis) is sufficiently long one may be able to study the erythropoietic growth curve before the bacteria become established.

If this is true, time and effort may be saved in not employing strict aseptic technique, but instead using sufficiently good technique so that the number of the bacteria does not become overwhelming in the period in which the cells are cultured. Such a procedure would make the whole technique more practical.

In 1940 Bullowa, Osgood, Bukantz and Brownlee (22), in the study of the effect of sulfapyridine on pneumococcus-infected marrow cultures found that in concentrations of about 10 milligrams per 100 milliliters, sulfapyridine did not appear to damage the marrow cells. Later, in 1951, Osgood was employing 100,000 units per liter of K salt penicillin G (97) in the nutrient medium to reduce the growth of possible contaminating bacteria. The sulfa and the antibiotic drugs may allow one to discard aseptic precautions almost completely in the culture of marrow cells, thus saving a great deal of time and labor.

Bone marrow cultures contain a large proportion of macrophages, monocytes and polymorphonuclear leucocytes. These cells doubtless play a big part in controlling the numbers of bacteria in these cultures and thus make the culture of bone marrow cells as a whole that much simpler.

4. Media

a. Diluting fluids

Two types of fluids are used in tissue culture; one type is the diluting fluid and the other

type is the nutrient fluid. Included in the diluting fluids which have been used in marrow cultures are Ringer's solution, Locke's solution, Tyrode's solution, and Gey's solution. Ringer's solution is unsatisfactory for mammalian cells because it is not buffered. Locke's solution contains no phosphates and therefore is limited in its buffering capacity. Tyrode's solution has been used successfully as a diluting fluid in bone marrow cultures by a number of workers including Carrell and Ebeling (26), Pierce (100), Rachmilewitz and Rosin (110), Armburst and Bett (10), and Norris and Majnarich (80). Gey's solution is similar to Tyrode's but has a higher potassium level, a lower calcium level and is not as highly buffered. The pH in this medium is controlled by equilibration with a five percent concentration of carbon dioxide in the atmosphere. Maintaining an atmosphere of five percent carbon dioxide involves replacing the air in the culture flask with a gaseous mixture containing five percent carbon dioxide. When one considers that this has to be done after every time that the culture flask is opened (such as after obtaining a sample for the counting of cells) it is readily seen that the extra work involved in using Gey's solution is a significant amount. Osgood (96) modified this medium by leaving out the bicarbonate and increasing the sodium chloride concentration to 8.0 grams per liter. In this way he was able to maintain a

Table I. Mammalian Physiological Salt Solutions

		Grams per 1000 cc. H ₂ O									
Solution		NaCl	KCl	CaCl ₂ Anhydrous	MgCl ₂ 6H ₂ O	NaH ₂ PO ₄ H ₂ O	Na ₂ HPO ₄ 2H ₂ O	KH ₂ PO ₄	MgSO ₄ 7H ₂ O	Glucose	NaHCO ₃
* Ringer		9.00	0.42	0.25							0.20
* Locke		9.00	0.42	0.24							
* Tyrode		8.00	0.20	0.20	0.10	0.05				1.00	1.00
+ Gey		8.00	0.37	0.17	0.21		0.15	0.03	0.07	1.00	0.227
** Gey		7.00	0.37	0.17	0.21		0.15	0.03	0.07	1.00	2.27
Osgood		8.00	0.37	0.17	0.21		0.15	0.03	0.07	2.00	

* After Cameron (25)

+ Used with atmospheric conditions

** Used with atmosphere containing 5% CO₂

Osgood (97)

pH suitable for the culture of marrow cells without having to replace the air above the fluid. However, Cameron (24) stated that NaHCO_3 is an essential constituent of any medium because it is important in the glycolytic activity of the cells. Therefore it seems desirable to use the medium recommended by Gey and Gey (56) for work under ordinary atmospheric conditions. This medium is the same as the first one that they proposed except that the NaHCO_3 concentration is reduced to one tenth and the NaCl is increased to 8.0 grams per liter. The compositions of all of the above media are given in Table I.

Sterilization of any solution containing the HCO_3 presents a problem because the HCO_3 breaks down to CO_2 and is blown off when the solution is autoclaved or boiled. This loss of CO_2 makes the solution more alkaline. These solutions are therefore best sterilized by filtration but even then CO_2 can be lost if the suction used, such as in Seitz filtration, is too great. They could also be prepared from sterile stock solutions by aseptic technique but this is perhaps somewhat hazardous.

b. Nutrient fluids

Plasma, reconstituted adult human plasma, serum, placental cord serum, embryo extract and liver

extract are among the various nutrient fluids that have been used in marrow cultures. Plasma has been used mainly in clot cultures and because of its fibrinogen content seems unsuitable for fluid cultures of marrow. Plum divided the serum which he used into two types. That serum which was from the same animal from which he obtained the bone marrow was called 'native' serum and serum taken from another animal of the same species was termed 'foreign' serum. Plum (104) found that on the average foreign serum gave about 15% poorer growth than did native serum. Osgood (89) used placental cord serum in many of his cultures as the nutrient medium. Cairns and Lajtha (23) used reconstituted dried plasma in the study of adhesion of leucocytes and obtained satisfactory growth with it. Carrel and Ebeling (26), Pierce (100), and Rachmilewitz and Rosin (110), used embryo extract in their bone marrow clot cultures and Gunz (63, 64) used it in his modification of Osgood and Brownlee's technique. The use of embryo extract entails a great deal of work in its preparation and there is much biological variation from one preparation to the next. Liver extract was found by Plum (104) to give fairly good growth when added to a suspension of marrow cells in Locke's solution. Thus it is seen that there are many nutrient fluids which could be used for marrow cultures but on the basis of the results of the above workers serum seems to give the greatest and most consistent growth.

5. Speed and Practicability

Should this project prove the feasibility of determining an erythropoietic inhibitor specific to malignant disease in such a way that it could be used in a diagnostic manner, the speed and practicability of the test would be of paramount importance. Therefore an attempt has been made in this project to keep techniques as simple as possible. Osgood and Brownlee's (88, 89) method of bone marrow culture is one of the simplest and yet most physiological marrow culture methods yet devised, and therefore recommends itself for the purposes of this project. The amount of equipment which is used is kept to a minimum, the cells are in suspension and therefore lend themselves readily to the addition of substances to be tested, the amount of growth can be determined fairly accurately, and bacterial contamination does not present a major problem. Therefore this method has been chosen, in principle, as the biological assay method whereby the presence of an erythropoietic inhibitor in malignancy can be tested.

SECTION V

APPARATUS, MATERIALS AND TECHNIQUE

By and large there is a fairly wide variation in the results of any biological research due to the inherent variability of the experimental material. This variability may be grossly exaggerated by even minor changes in technique. Accordingly this section on apparatus, materials and technique is given in detail.

A. Apparatus

1. Autoclave

Prometheus type 1400. Electrically operated with steam pressure up to 22 pounds per square inch.

2. Beakers

Pyrex glass; assorted sizes.

3. Capillator set for determining pH

Set of sealed capillary tubes with solutions of phenol red ranging from pH 6.8 - 8.4.

4. Centrifuges

a. International size (serial number 5-335) with combination 15 ml. and 50 ml., eight place, 90° head.

b. International clinical model (serial number 5-102) with four place, 90° swing combination 15 ml. and 50 ml. head.

c. Chicago Surgical and Electric Company type 91 (serial number 1923) with four place, 90° swing, 15 ml. head.

5. Centrifuge tubes

a. 15 ml., Kimble brand, pyrex, ungraduated, conical bottom, centrifuge tubes with cork stoppers or custom made teflon caps.

b. 15 ml., pyrex, graduated, conical bottom centrifuge tubes with corks or caps similar to above.

c. 50 ml., pyrex, ungraduated, round bottom centrifuge tubes with neck fitted with size 00 rubber stoppers.

d. Wintrobe, hematocrit tubes (new model) with rubber caps.

6. Cloth pipette covers.

Double thickness, linen cloth bags in which 0.1, 1, 2, 5 and 10 ml. measuring and transfer pipettes were placed for sterilizing.

7. Colorimeter

Fisher AC model electrophotometer (serial number 7-101).

8. Counter

Hand tally used in recording the number of cells counted during a cell count.

9. Counting chamber

Spencer bright-line hemocytometer with improved Neubauer double ruling. Hellige hemocytometer cover-glasses, rectangular in shape 20 x 26 mm. and 0.4 mm. thick.

10. Cover-glass clips

German silver cover-glass clips modified to fit above hemocytometer and cover-glasses.

11. Culture bath

Fisher unitized constant temperature bath with ether and mercury thermostat of the vapor type, motor stirrer unit, heater unit and thermometer. See figure I.

Figure I



Culture Bath

12. Culture bottles

250 ml. round bottomed lead-free glass centrifuge bottles fitted with size 6 rubber stoppers.

13. Drying oven

10" x 12" x 10" size, thermostatically controlled, gravity convection with temperature range 30° C. to 180° C.

14. Glass rods

Pyrex glass rods used to free the fibrin in clotted plasma.

15. Graduated cylinders

10, 25, 50, 100, 500 and 1000 ml. sizes. Used for measuring liquids and the larger ones were used for soaking and cleaning volumetric and transfer pipettes.

16. Homogenizer

A flat-bottomed, pyrex, 15 x 80 mm. test tube with a snugly fitting plunger made of a glass rod and a rubber washer made from a piece of rubber tubing. See figure II.

17. Instrument sterilizer.

Electrically heated, boiling water instrument sterilizer.

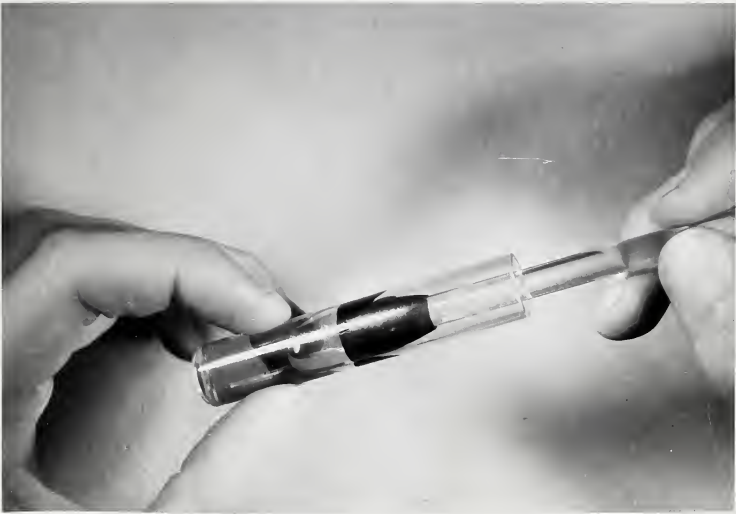
18. Micro slides

25 x 75 mm. non-corrosive microscope slides.

19. Microscope

Spencer, binocular microscope with 16 mm., 4 mm. and 1.8 mm. objectives and mechanical stage.

Figure II



Homogenizer

20. Needles

a. B-D Yale, Luer-lok, regular point, stainless hypodermic needles Gauge - 26, 25, 22, 20 and 18.

b. Bone marrow aspirator needle. Gauge 16 hypodermic needle of above type modified by grinding the tip off so as to give a blunt, flat end.

21. pH meter

Coleman pH electrometer.

22. Pipettes

a. Red cell blood diluting pipettes No. 1552, F & B Precision with rubber tubing and mouth piece.

b. White cell blood diluting pipettes No. 1551, F & B Precision.

c. 0.1 ml., 0.2 ml., 1.0 ml., 2.0 ml., 5.0 ml., and 10.0 ml., graduated measuring pipettes.

d. 10 ml. volumetric pipettes.

e. Ungraduated transferring pipettes of various sizes and bore. Made of pyrex glass.

23. Pipette cleaner

Fisher pipette cleaner for washing and drying 16 blood diluting pipettes at once. Consists of an aluminum head with a serrated hose connection which fits over a large rubber pipette holder having 16 holes.

24. Pipette shaker

Electric pipette shaker for blood diluting pipettes which has a shaking action composed of about 1200 rapid, transverse vibrations of small amplitude

extending over a period of about 30 seconds after which time it automatically shuts off.

25. Portable power saw.

Dremel moto-tool with circular saw blade.

26. Power-driven brush

2" nylon brush powered by a variable speed electric motor.

27. Receiving flask

Stout-walled, Erlynmeyer flask with side arm.

28. Rubber adaptors

Used to fit the Seitz filter to the receiving flask.

29. Seitz filter

Seitz combination Uhlenhuth - Mantenfel model size 6 100 ml. capacity filter made of silver metal. Filter pads are of asbestos 60 x 30 mm. and of 3 pore sizes - 0.1 micron, 2 microns and 5 microns.

30. Stills

a. Water still. Tin-coated copper, automatic electric still.

b. Water still. Consists of a Friedrich's pyrex glass condenser with spiral inner tube and pyrex glass Claissen distilling flask with two necks.

c. Alcohol still. Friedrich's pyrex glass condenser with spiral inner tube and a straight distilling column connected to a boiling water bath heated Florence flask.

31. Stock bottles

1000 ml. capacity pyrex stock bottles with outlet at the bottom.

32. Surgical instruments

An assortment of scalpels, scissors, forceps, probes, hemostats, bone forceps and needles.

33. Syringes

a. B-D Yale tuberculin syringe (1 ml.) with blue plunger.

b. B-D Yale 2, 5, 10, 20 and 30 ml. sizes made of resistance glass.

c. B-D Yale Luer-lok, 30 ml. size syringes made of special resistant glass.

34. Test tubes

a. 20 x 155 mm. used as containers for blood diluting pipettes. Stoppered with non-absorbant cotton plugs.

b. 25 x 150 mm. test tubes used as containers for sterilizing Wintrobe tubes, Wintrobe pipettes, homogenizer, needles, tuberculin syringes and glass rods.

35. Vacuum pump

Powered by one-sixth horsepower electric motor.

36. Wash bottles

Polyethylene plastic bottles with glass tube outlet.

B. Miscellaneous Expendable Material

Various materials used in the course of these experiments are listed under Appendix I.

C. Biological Material.

1. Blood

a. Rabbit - obtained by femoral or carotid arterial puncture.

b. Rat - obtained by heart puncture or from cut end of tail.

c. Human - obtained by venipuncture from Mr. McLuhan or Mr. Nelson.

2. Bone Marrow

a. Rabbit - obtained from shaft of right femur; always contains a fairly large percentage of peripheral blood.

b. Rat - obtained by aspiration of shafts of femurs or humeri.

3. Bone Marrow Culture Stock.

This is the solution of bone marrow cells in their final concentration of about 1000 - 3000 per cu. mm. before culturing begins. This solution is divided into 15 ml. culture volumes.

4. Concentrated Bone Marrow Suspension

The concentrated stock solution of the myeloid-erythroid layer of cells in the diluting fluid (with or without serum) on which the first nucleated count is made to determine the dilution necessary to give a concentration of nucleated cells between 1000 and 3000 per cu. mm.

5. Rats

Adult white rats of either sex.

6. Rabbits

For the most part these were adult female, domestic white or brown rabbits which had been previously used by the Department of Pathology, Provincial Laboratory for Friedman pregnancy tests and which had been ovariectomized at least two weeks previous to the time that they were used as a source of blood and bone marrow. The occasional adult male rabbit was used as well.

7. Serum

Rabbit or human obtained by immediate centrifuging of the blood 1500 - 2500 r.p.m. for 15 minutes to separate the fibrin from the serum.

D. Cleaning and Sterilization of Equipment

All glassware, needles and instruments were rinsed in hot running tap water as soon as possible

after being used. The instruments were allowed to soak in calgonite solution for a few minutes, rinsed and dried with a towel. They were sterilized by dry heat (160° C. for 90 minutes) or in the electric boiling water sterilizer before the next operation.

Syringes, needles, centrifuge tubes, glass rods, Wintrobe tubes and pipettes and homogenizer were soaked for a variable period in calgonite solution and then boiled in the same solution for 20 minutes. After being rinsed five to ten times in running hot tap water they were rinsed about five times in distilled water and then soaked for an hour or more in distilled water. They were then transferred to ethyl alcohol and allowed to stand in it for 3 hours or overnight after which they were rinsed five times with distilled water and allowed to dry at room temperature. Such glassware as Wintrobe tubes and pipettes were given a final acetone rinse in order to facilitate drying. When dry the syringes were wrapped (plunger and barrel separate) in brown wrapping paper, and the needles, glass rods, Wintrobe tubes and pipettes and homogenizer were placed in test tubes stoppered with cotton plugs. Because rubber is toxic to bone marrow cells (Gunz, 63) the plunger of the homogenizer was dipped before autoclaving in melted paraffin about every second time it was used.

The ungraduated pipettes, and the transferring, measuring and volumetric pipettes were not boiled in calgonite but otherwise were cleaned in the above manner.

The final rinse was made with acetone after which the pipettes were dried by sucking air through them. They were then wrapped in brown paper or placed in linen cloth bags before autoclaving.

The blood diluting pipettes were placed in the pipette cleaner attached to a water vacuum pump, rinsed a couple of times in tap water, washed with calgonite solution, rinsed in tap water again then distilled water, and finally acetone after which they were dried by sucking air through them. They were prepared for autoclaving by placing them in test tubes stoppered with cotton plugs.

The culture bottles were first rinsed out with hot running tap water and then filled with calgonite and allowed to stand overnight. As a further precaution against the presence of organic material in these bottles it was felt that some mechanical cleaning of them was necessary and accordingly the power-driven nylon brush was used to brush them out thoroughly with calgonite solution after which they were rinsed ten times in hot running tap water, five times with distilled water and then filled with 95% alcohol and allowed to stand overnight. Again they were rinsed five times with distilled water and then soaked in distilled water for a variable period before autoclaving. Towards the end of the project some of the culture bottles were siliconed in an attempt to prevent

nucleated cells from sticking to the inside of the glass (Cairns and Lajtha (23)). After the above cleaning they were rinsed with Dri-film and then distilled water. Excess Dri-film was wiped from the side of the culture bottles by winding cloth around the power-driven nylon brush and brushing the inside of the bottles. On completion of this they were soaked in alcohol and rinsed with distilled water as above.

If any of the above pipettes, centrifuge tubes or culture bottles contained excessive organic material which was not easily rinsed off they were soaked overnight in cleaning solution, rinsed well in hot running tap water and then cleaned in the manner described above.

The rubber stoppers were soaked in tap water after use, boiled in distilled water for about an hour and then soaked in fresh distilled water until they were autoclaved.

The centrifuge tubes were usually sterilized by dry heat (160° C. for 90 minutes) in the drying oven instead of being autoclaved. It was noticed that there was often some condensation in them after autoclaving. The rest of the glassware and needles were sterilized by autoclaving at 20 pounds pressure for 20 to 30 minutes. The pressure was released slowly at the end of this time and the equipment was left in the autoclave with the door partially open until the autoclave had cooled down to

room temperature. The rubber stoppers and teflon caps for the centrifuge tubes were autoclaved and then placed in the necks of the centrifuge tubes (using aseptic technique) after they had been sterilized and had cooled down to room temperature.

E. Preparation of Diluting Fluids

Stock solutions of the various salts necessary for the preparation of the different diluting fluids were made up in distilled water in the concentrations given in Table II. These solutions were autoclaved, brought back to volume with sterile distilled water and stored in sterile stock bottles.

During the first part of the project a Seitz filter was not on hand and accordingly the diluting fluids had to be sterilized by autoclaving. The Gey's solution which is equilibrated with an atmosphere containing 5% carbon dioxide was prepared in the following manner. The given amounts of the salt stock solutions were added to about 700 ml. of distilled water in a 1000 ml. volumetric flask with the exception of the calcium chloride. The solution was thoroughly shaken after the addition of each salt. The salt mixture, a separate quantity of calcium chloride stock solution and a few hundred milliliters of distilled water were autoclaved. The calcium chloride

Table II.* Composition of Gey's Solution

Constituents	Concentration		Stock Solutions		Quantities for + B.S.S.
	gm./l.	Mol. conc.	gm./l.	ml. per liter	
NaCl	7.0	5.0	272.25	19.2	
KCl	0.37	1.0	74.55	5.0	
NaHCO ₃	2.27	1.0	84.01	27.0	
CaCl ₂ (anhyd.)	0.17	0.1	11.1	13.5	
MgCl ₂ x 6H ₂ O	0.21	1.0	203.33	1.0	
Na ₂ HPO ₄ x 2H ₂ O	0.15	0.5	89.03	1.6	
KH ₂ PO ₄	0.03	1.0	136.14	0.2	
MgSO ₄ x 7H ₂ O	0.07	0.1	24.65	3.0	
d - glucose - 1.0 gm. - weigh out fresh					

For medium used under ordinary atmospheric conditions use 1/10 the amount of NaHCO₃ and increase the NaCl to 8.0 gm. per liter.

* After Gey and Gey (56)

+ Balanced salt solution

solution was brought back to volume with the sterile distilled water and then the amount given in Table II was added to the salt mixture.

It was found that the salt solution developed a fine white precipitate when calcium chloride was added to it before autoclaving. Dextrose is thought to caramelize during autoclaving and is therefore added afterwards.

The given amounts of sterile dextrose powder and sodium bicarbonate were weighed out aseptically and added to the salt solution after which sterile distilled water was added to bring the volume to 1000 ml. After thorough shaking the solution was transferred to a sterile 1000 ml. stock bottle.

Osgood's solution was prepared in the above manner according to Table III but no bicarbonate was added.

Table III*: Composition of the Culture Medium

Balanced Salt Solution			
NaCl	8.00 g.	$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	0.15 g.
KCl	0.37	KH_2PO_4	0.03
CaCl_2 (anhyd.)	0.17	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.07
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.21	Dextrose	2.00

* After Osgood

Table IV. Stock Solutions for Gey's Solution

Constituents	Concentration		Stock Solution		Quantity for B.S.S.
	gm./l.	Mol. conc.	gm./l.	ml. per liter	
NaCl	7.5	5.132	300	25	
KCl	0.37	0.4963	37	10	
NaHCO ₃	0.227	0.2698	22.7	10	
CaCl ₂ (anhyd.)	0.17	0.1532	17	10	
MgCl ₂ x 6H ₂ O	0.21	0.1033	21	10	
Na ₂ HPO ₄ x 2H ₂ O	0.15	0.1056	15	10	
KH ₂ PO ₄	0.03	0.0220	7	10	
MgSO ₄ x 7H ₂ O	0.07	0.0284	7	10	

d - glucose - 2 gm. - freshly weighed out.

Midway through the project new salt stock solutions were prepared using double distilled water. The concentrations of these are given in Table IV. At this time a Seitz filter was obtained allowing solutions containing the bicarbonate ion to be sterilized without it being lost in the form of carbon dioxide as happens when such solutions are autoclaved. Therefore from this time on the Gey's solution suitable for use under atmospheric conditions was used. The salts and the dextrose were added to about 700 ml. of double distilled water in the amounts given in Table IV. After thorough mixing double distilled water was added to the 1000 ml. mark and the solution was filtered through a freshly autoclaved Seitz filter into a sterile receiving flask. The filtration was done under reduced pressure in the receiving flask obtained with a water vacuum pump. If the suction in the receiving flask is too great there will be an excessive loss of CO_2 . Therefore as little suction as possible was used in filtering this solution. A 0.1 μ pore size filter disc was used and was thoroughly rinsed with distilled water after use. The salt mixture was transferred to and stored in a sterile 1000 ml. stock bottle. Two hundred thousand units of crystalline penicillin - G, potassium was mixed up in sterile double distilled water and added to the salt solution in order to inhibit growth of at least some of the bacteria which

may have gained entrance to the mixture. Towards the end of the project this salt solution was stored in the refrigerator to further reduce growth of possible contaminating bacteria or fungi.

If the pH of any of the above balanced salt solutions was not within the range pH 7.2 - 7.5 it was adjusted to that range with sterile decinormal NaOH or HCl.

F. Technique of Obtaining and Culturing Bone Marrow Cells

On the day that was set aside for culturing, work was begun about 8:00 or 8:30 A.M. A rabbit was weighed and placed on the operating table. A solution of nembutal was then mixed up by dissolving 200 mgm. (contained in a capsule) of nembutal powder in 4 ml. of distilled water. The dose was 30 mgm. per kilo given by ear vein using No. 26 needle. It was found that this procedure did not require placing the rabbit in an anesthesia box, nor was transillumination of the ear necessary to show up the marginal vein. This amount of nembutal markedly reduced the muscle tonus but breathing was maintained at an adequate level and the rabbit was still sensitive to pain. While the full effect of the nembutal was taking place the hair on the anterior aspect of the neck was clipped and the area was swabbed

with 70% alcohol or zepherin chloride. A tray of sterile instruments, syringes, needles, centrifuge tubes and the portable power saw were arranged near the operating table in readiness for the operation.

Open drop ether was administered until the respirations became deep and steady and the rabbit insensitive to pain. The operator scrubbed but did not use gloves. A midline incision 7 - 10 cm. long was made with sterile scissors over the anterior aspect of the neck. Then using a fresh set of sterile forceps and scissors the wound was extended more deeply by dissecting through the adipose tissue, fascia and muscles down to the trachea. Dissecting to the right or left of the trachea one of the carotid arteries was exposed and dissected free for a distance of about 3 - 5 cm. The anesthetist then clamped the artery with a hemostat distal to and a bulldog proximal to the site of puncture. Holding the artery steady with a sterile pair of forceps the artery was punctured between the hemostat and the bulldog using a sterile 30 cc. syringe and No. 18 gauge needle. The needle was aimed proximally with the bevel up during this procedure. As soon as the needle had been inserted about 1 - 2 cm. along the lumen of the artery the bulldog was released and then clamped over the part of the needle that was in the artery as a precaution against its slipping out. After 30 - 35 cc. of blood had been withdrawn the artery was clamped with a hemostat by the anesthetist proximal to the puncture. The syringe was then quickly withdrawn and the blood run into two sterile, 15 ml. ungraduated centrifuge tubes from which

the anesthetist had just removed the caps. Replacing the caps the centrifuge tubes were balanced by adding water to the retaining cups, placed in the centrifuge and spun at 2000 r.p.m. for 15 minutes. This separated the plasma from the red cells. However, by this time the plasma had clotted so that the fibrin had to be freed from the walls of the tube with a sterile glass rod. The tubes were then replaced in the centrifuge and spun for another 15 minutes at 2000 r.p.m. to pack the fibrin on top of the red cells thus giving a clear layer of serum on top.

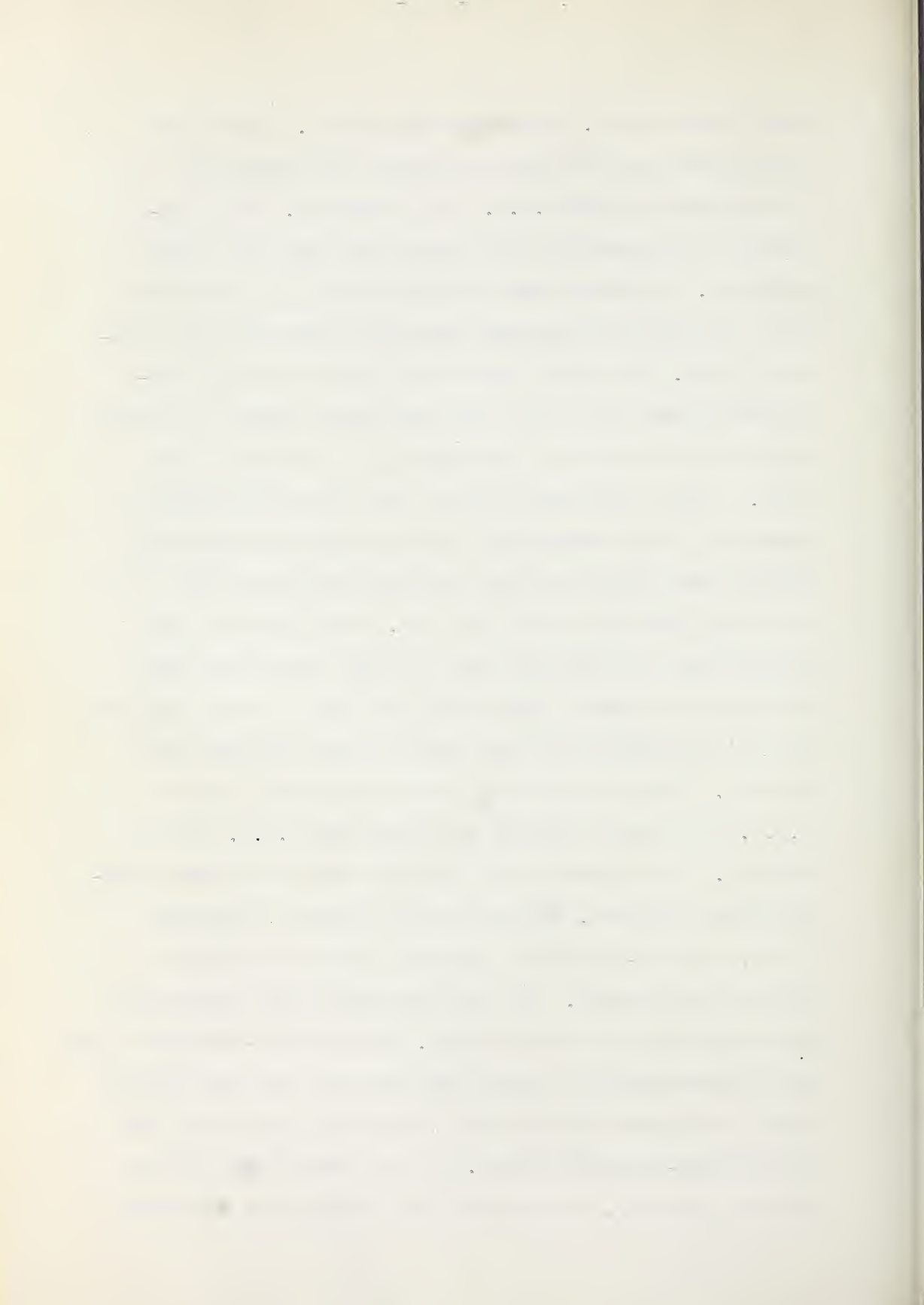
During this time the operator had clipped the hair over the right thigh and sterilized this area with alcohol or zepherin chloride. The anesthetist (McLuhan) then became the operator and the former operator (Nelson) became his assistant. The operator made a lateral incision over the right thigh extending from the knee to above the greater trochanter. The femur was exposed by dissecting between the anterior and lateral vasti. The muscles attached to the greater trochanter were severed and the head of the femur severed from all its connections. Placing a pair of bone forceps on the greater trochanter and exerting an upward pull the femur was freed of all its surrounding muscle and tissue distal to the knee. The shaft of the femur was scraped free of all tissue using a fresh sterile scapel. Placing bone forceps on the distal and proximal ends of the femur to stabilize it the portable power saw with a sterile saw blade was used to saw the femur in half. Two or three ml. of Gey's solution were

drawn into a 30 cc. syringe from a 15 ml. quantity in a 15 ml. centrifuge tube. Then the bone marrow (which was very red in the shaft of the rabbit femur as opposed to the yellow marrow in those of larger animals) of both halves of the femur was aspirated into the syringe using the aspirator needle. This material was placed in the homogenizer. The syringe was rinsed out once with about 1 ml. of Gey's solution which was then added to the homogenizer.

By this time the rabbit's blood volume had been partially restored by the transfer of extra-vascular fluid to the vascular tree. Consequently another arterial puncture yielding about 30 - 40 cc. of blood could be made again. Usually the other carotid artery was used for this puncture. The blood was treated in the same manner as the first sample in order to obtain the serum from it.

After the second arterial puncture the bone marrow was homogenized. This was accomplished by inserting the plunger in the homogenizer (see figure II) and working it back and forth with a slow, rotating movement. The lumps of cells were squeezed between the rubber washer and the inside of the test tube and this way separated into smaller clumps. This process was continued until the mixture took on a homogenous appearance. This material was then added to the quantity of Gey's medium

left in the 15 ml. centrifuge tube above. Replacing the cap on the tube the bone marrow suspension was centrifuged at 1500 r.p.m. for 15 minutes. This separated the contents of the centrifuge tube into three portions. Uppermost was the fatty layer, in the bottom were the cells and between these two layers was the diluting fluid. The fatty layer was pipetted off and discarded as well as all of the supernatant except a quantity equal to the volume of the cells in the bottom of the tube. Using a Wintrobe pipette the cells were evenly suspended in the remaining diluting fluid by repeatedly sucking the cells and fluid up into the pipette and squirting them back into the tube. This material was transferred to a Wintrobe tube and the centrifuge tube and Wintrobe pipette were rinsed out with a small quantity of Gey's solution which was added to the Wintrobe tube as well. The Wintrobe tubes were centrifuged at 500 r.p.m. for about 5 minutes and then 1500 r.p.m. for 10 minutes. The layers in the Wintrobe tube from above downward were (Limarzi, 76) yellow fat, red fat, diluting fluid, myeloid-erythroid layer and on the bottom the mature erythrocytes. The fat and most of the supernatant were pipetted off and discarded. The myeloid-erythroid layer was transferred to a second Wintrobe tube and centrifuged again to separate further the mature red blood cells and the myeloid-erythroid layer. In the latter part of the project, however, this second centrifuging was dispensed



with in order to save time. The myeloid-erythroid layer was placed in a 15 ml. centrifuge tube containing about 5 ml. of Gey's solution. Using a Wintrobe pipette and repeatedly sucking the cells and fluid up into the pipette and squirting them back into the centrifuge tube the cells were evenly suspended in this diluting fluid.

Employing a sterile white blood cell diluting pipette a nucleated cell count was done using a 1:20 dilution with 1% acetic acid. In this way the number of cells per cubic mm. was determined and the dilution factor necessary to give a final concentration of 1500 - 3000 nucleated cells per cubic mm. in the cultures was calculated.

The culture medium was prepared by mixing one part serum (prepared previously as above) and two parts diluting fluid. About 75 ml. of medium was prepared if four cultures of 15 ml. each were to be incubated. Using the dilution factor calculated above a quantity of the concentrated bone marrow suspension was added to the medium. The resulting suspension was thoroughly mixed and transferred to four culture bottles in the following manner. The culture bottles were named A, B, C, and D. With a 5 ml. graduated pipette, 5 ml. aliquots were withdrawn (while shaking the bottles containing the suspension continuously) and transferred to the flasks in this order A, B, C, D, D, C, B, A, A, B, C and D.

It was found early in the project that dividing the bone marrow suspension into four cultures by merely pipetting out one quarter of the original volume into four successive culture bottles resulted in wide variations between the cultures in concentrations of the cells. The above procedure gave more equal cell concentrations in all the cultures.

At this juncture the material to be tested was added to 2 of the cultures and the other two were used as controls. Aseptic technique was observed throughout the above.

From each culture bottle a red cell blood diluting pipette-full of the culture material was withdrawn after swirling the bottle by hand for about one minute to insure even suspension of the cells. At the same time a quantity from each bottle was drawn up to the 1 mark in four white cell diluting pipettes. Each of these quantities was then diluted 1:20 with 1% acetic acid. The four cultures were suspended in the constant temperature water bath and incubated at 37.5° C. During the next two hours counts were made on the undiluted material in the red cell pipettes and on the diluted material in the white cell pipettes by the usual method of counting cells in a hemocytometer discussed in a previous section. In this way total and nucleated cell counts were obtained on each culture at zero time. Total and nucleated cell

counts were also made at 2, 4 and 6 hours. The pipettes were filled from the cultures at the same time so that the culture bottles were out of the bath at the same time and for the same period (usually 10 - 15 minutes). The counts were calculated in terms of percentage (zero hours being 100%) and graphed. The difference between the total and nucleated cell counts is the non-nucleated or mature red cell count. This was also expressed as a percentage and graphed.

One worker did all the counting one day while the other worker cleaned and prepared the equipment for the next series of cultures. On the morning following the day that the cultures had been incubated the contents of the red cell pipettes, which had been kept overnight in the refrigerator were emptied individually on a watch glass and transferred to Wintrobe tubes. These tubes were centrifuged at 1500 r.p.m. for about 15 minutes and all the supernatant except a quantity equal to the volume of cells in the bottom of the tubes was withdrawn and discarded. The cells were suspended in the remaining diluting fluid and a smear was made of this on a clean glass slide. These slides were marked with the culture bottle numbers and the time of incubation and then stored away for future reference. Ideally, these smears should have been made immediately after the counts had been done. However, there was not adequate time to do this in between

successive counts. Therefore the above procedure was followed realizing that changes could take place in the cell picture in the time that the pipettes were in the refrigerator. In spite of this these smears will have some value should they be referred to in the future.

In some of the experiments liver extract or some other fortifying substance was used in place of serum. In these cases the medium consisted of Gey's or Osgood's solution alone and the correct quantity of concentrated bone marrow suspension necessary to give a concentration of 1500 - 3000 nucleated cells per cubic millimeter was added to this medium. This master suspension was then divided into four cultures in the manner described above. The substance to be tested was added at this juncture and the rest of the procedure followed was as above.

In other experiments where serum was added to some cultures and liver extract, Amigen or B₁₂ to others as in rabbit No. 27 the medium for each individual culture was prepared separately in the culture bottles so that there was the same total quantity of medium (including the substance to be tested) in each culture bottle. Then the same quantity of concentrated marrow cell suspension was added to each culture bottle to give a concentration of 1500 - 3000 nucleated cells per cubic millimeter.

At the beginning of the project a number of cultures was made in which Gey's solution equilibrated with 5% carbon dioxide was used. The medium was equilibrated with an atmosphere of 5% carbon dioxide and 95% oxygen (carbogen). The cells were added to the culture and the atmosphere in the bottles was replaced with carbogen again. This was accomplished by inserting 2 sterile needles through the rubber cork on a culture bottle and allowing carbogen (which had been run through a sterile cotton filter) to replace the air in the bottle. This procedure had to be performed after each use of the culture bottle.

This medium was replaced by Osgood's and later by the Gey's solution suitable for work at atmospheric conditions for two reasons. Firstly, replacing the air in the culture bottle with carbogen was time consuming and secondly it was thought that the high concentration of oxygen might have a depressing action on the bone marrow cells even if such is at variance with the results of Rosin and Rachmilewitz (111).

SECTION VI

RESULTS

The results of 101 bone marrow cultures are presented. Ninety-four of these cultures were of rabbit bone marrow and 7 were of rat bone marrow.

The actual cell counts at various intervals made on these cultures are presented in table form in Appendix II. The results are presented in graphic form in Figures III to XXIII of all the cultures except those of rat bone marrow and those in which the cultures were followed over a period of about 24 hours. The percentage values based on 100% at zero hours of the total cell counts,

nucleated cell counts and non-nucleated cell counts (red cell counts) are given in these graphs. The non-nucleated cell count is the difference of the total and nucleated cell counts.

A. Initial Series of Cultures

A preliminary series of cultures was carried out to standardize the technique of the chosen method of bone marrow culture. Emphasis was not placed on obtaining results in these cultures and accordingly the results of this series are not included.

B. Duration of Culture Period

It was desirable to determine early in the project the period during which the most marked multiplication of cells took place. A series of 12 cultures (8A - 13B Appendix II) was followed over a period of about 24 hours with this end in view. The culture medium in these cultures was 67% Osgood's fluid and 33% homologous serum.

In cultures 8A and 8B the total cell count rose to its highest level at the end of 7 hours. The nucleated cells gradually increased over the 24 hour period and had a higher relative increase than either total or non-nucleated cells. In cultures 9A and 9B the total and non-nucleated cells reached a peak at the end of 12 hours

after which they dropped off. The nucleated cells with some minor fluctuations above the 100% level fell off during the culture period. In cultures 10A and 10B there was an initial rise in the total cells in the first 8 hours (more marked in 10A) which was maintained or slightly increased over the next 16 hours. The nucleated cells were maintained at a constant level in 10A and increased markedly in 10B by the twentieth hour. The non-nucleated cells reacted in an opposite manner to that of the nucleated cells (i.e., an increase in 10A and relatively little change in 10B). Cultures 11A, 11B, 12A and 12B show much the same changes; namely, an initial increase followed by a decrease in the total and non-nucleated cell counts and an initial decrease in the nucleated cells followed by a persistence at approximately this new level. The total cells in 13A acted in an almost opposite manner to those in 13B. The nucleated cell counts however showed an initial increase in the first 6 hours followed by a slight decline.

The overall interpretation of these results was that the major increase in cells (usually non-nucleated cells), when it occurred came in the first few hours of incubation. Therefore it was decided for technical reasons to restrict the culture period in subsequent cultures to five or six hours since the purpose of this project was to obtain the greatest possible growth in a short period of time and not to follow the course of the cells over as long a period of time as possible.

C. Effects of Osgood's and Gey's Fluids each Reinforced with Homologous Serum.

Osgood (89, 90) and Plum (104) both recommend diluted serum as a good if not the best nutrient material for bone marrow cultures. Accordingly it was decided to incubate a series of cultures first using homologous serum diluted with Osgood's medium and then homologous serum diluted with Gey's medium. Figures III - V give the results of cultures using Osgood's fluid and serum and Figures XIV, XXI (Rabbit 36) and XXIII give the results of cultures in which Gey's fluid fortified with serum was used.

1. Cultures with Osgood's Fluid and Serum

With the exception of 16A and 16B the culture medium in all this series was composed of 67% Osgood's fluid and 33% homologous serum. In 16A and 16B the proportions were 90% and 10% respectively.

Marked increases in the non-nucleated cells in 14B and 18A, less marked increases in 14A, 15B and 17A, small increases in 16A, 16B, 17A and 19A, minor fluctuations from the 100% level in 15A and 19B and a definite decrease in 18B were observed. The changes in the total cell counts were for the most part similar to the changes in the non-nucleated cell counts but to a lesser degree. The nucleated cells decreased in 14A, 15A, 15B, 16A, 17A, 17B and 18A and possible increases in 14B, 16B, 18B, 19A and 19B of the nucleated cells were observed. The high 2 hour

nucleated cell count in 19B is probably a counting error.

The growth in 16A and 16B is not as marked as the growth in some of the other cultures.

These results indicate that definite cell growth usually occurred in serum-fortified Osgood's medium. This growth was made evident by the increase in total cell population. The reduction of nucleated forms which occurred, in the face of a total cell increase introduced the interesting phenomenon which was to be noted frequently; viz., the reciprocal changes that appeared to occur between nucleated and non-nucleated forms. This point is discussed later.

The data from these experiments do not permit conclusions to be made concerning the relative merits of a 10% and 33% serum concentration.

2. Cultures with Gey's Fluid and Serum

Figures XIV, XV, XVII, XVIII, XIX, XX, XXI, XXII and XXIII illustrate cultures 28 A, 28 B, 28 C, 28 D, 29 A, 29 C, 31 C, 31 D, 32 A, 32 C, 33 A, 33 B, 34 C, 34 D, 35 A, 35 B, 36 A, 36 B, 37 A, 37 B, 38 A and 38 B which were all cultured in a medium of 67% Gey's fluid and 33% homologous serum.

A very good increase in the number of non-nucleated cells was obtained in cultures 29A, 29C, 32C, 33A, 36A and 37B, a moderate increase was obtained in 28B, 28 D, 31C, 32A, 33B, 34C, 35A, 35B, 36B, 38A and 38B, a very slight increase was observed in 28C, 31D and 37A and a decline or doubtful increase was seen in 28A and 34D. Thus there were only 2 cultures of these 22 in which there was not an apparent increase in the number of red cells.

The total cell counts corresponded very closely to the non-nucleated cell counts except that the changes seen in them (total cells) were not so marked as in the non-nucleated cells.

Cultures 28C, 31D and 38A are the only three cultures in this series where a decrease in the nucleated cell count was not fairly definite. In the rest of the cultures there were fairly definite decreases of varying degrees in the nucleated cells over the 6 hour period. In the three cultures mentioned as well as 34D, 37A, 38A and 38B where the nucleated cell count does not diminish (or at least not very markedly) it is noted that there is not a large increase in the non-nucleated count. In all of the cultures in which there was a good increase in the non-nucleated cell count there was an accompanying definite decrease in the nucleated cell count. This point was also noted in the previous section.

In comparing the results of these cultures which employed Gey's fluid in place of Osgood's it is seen that while the increases in non-nucleated cells in cultures using Gey's fluid were never of the order of those seen in 14B and 18A which were cultured in Osgood's fluid nevertheless there was a greater degree of consistency of growth (and also of the decreases in the nucleated cells) in those cultures employing Gey's fluid.

D. Effect of Liver Extract as Compared with Effect of Osgood's Fluid Alone.

Figures VI, VII, VIII, IX and X illustrate cultures 20A, 20B, 21A, 21B, 22A, 22B, 23A, 23B, 24A and 24B which were performed in Osgood's fluid alone. With the exception of 23A, 23B and 24B there was a definite decrease in all the cells with the greatest relative decreases being in the nucleated cell counts and the smallest in the non-nucleated cell counts. All the cells in 24B decreased in concentration but here the nucleated cells did not decrease in number as quickly as the non-nucleated cells. In 23A there was a slight increase in the non-nucleated cells and a moderate drop in the nucleated count. In 23B there was a moderate increase in the non-nucleated cells and a marked fall in the nucleated cells.

From these results it appears that unfortified Osgood's fluid is a poor bone marrow culture medium.

In cultures 20C and 20D where 0.067 U.S.P. units of liver extract per ml. of culture medium was added all the cells and especially the nucleated cells were maintained at higher levels than they were in the control cultures (i.e., without liver extract) 20A and 20B. In 21 C and 21D where the concentration of liver extract is ten times the above only the nucleated cells were maintained at a higher level than were those in the controls. In 22D where the concentration is only one tenth that of 20C and 20D the non-nucleated cells were perhaps maintained at a higher level than those in the controls. The nucleated cells perhaps dropped off slightly more in 22C and 22D than in the controls (i.e., without liver) but otherwise little difference was seen between these cultures. The concentration of liver extract in 22C and 22D was 0.0067 U.S.P. units per ml. of culture medium or 1/10 of that in 20C and 20D. In 23 C and 23 D where there was 0.15 U.S.P. units of liver extract per ml. all the cells were maintained at a higher level than were those in the controls. Definite increases in the non-nucleated and total cell concentrations took place. However, in 24C and 24D where the same concentration of liver extract was used it appears that only 24C showed

better growth (in terms of non-nucleated cells) than that seen in the controls. The growth curves of the cells in 24D are very little different from those of 24A and 24B.

A review of all the graphs comparing growth in Osgood's medium alone, with growth in Osgood's medium fortified with liver extract, indicates a definite and beneficial effect of the liver extract on the cultures. But it is not possible to draw conclusions regarding the optimum concentrations of liver extract.

E. Effect of Amigen and Liver Extract

Osgood's fluid alone contains no protein or amino acids from which new cells can be made. Amino acids in the form of Amigen were used to fortify Osgood's fluid in 25B, 26B and 27B and the effect was compared with the response obtained in control cultures 25A, 26A and 27A in which the medium was 67% Osgood's fluid and 33% homologous serum. Figures XI, XII and XIII illustrate these results.

In 25B where 0.067 ml. of Amigen per ml. of culture medium was added the nucleated cells fell off very little in number and compared favorably with the control culture (25A). The non-nucleated and total cells on the other hand fell off sharply as compared to those in 25A.

The nucleated cells in 26B where 0.01 ml. Amigen per ml. of culture medium was added decreased in number somewhat more rapidly than those in 26A and similarly with the non-nucleated and total cells in 26A. The nucleated cells in 27B where the concentration of Amigen was the same as that in 26B seemed to increase more than the nucleated cells in 27A while the non-nucleated and total cells followed roughly the same course in the 2 cultures; namely, a slight to moderate decrease.

A comparison of the graphs showing the effect of Amigen in comparison with control cultures in Osgood's medium indicates a deleterious influence of the Amigen.

The effect of Amigen and liver extract combined in Osgood's fluid was also tested in this series; namely, in 25C, 26C and 27D. In 25C there was about the same decrease in nucleated cells as that observed in 25B (Amigen and Osgood's). Here the 0.067 U.S.P. units of liver extract per ml. of culture medium was added to the same concentration of Amigen as in 25B. The decrease in the non-nucleated and total cells was not as great as in 25B but there was also no increase in them as was observed in 25A.

0.15 U.S.P. units of liver extract per ml. of culture medium was added to 26C and 27D where the Amigen concentration was the same as in 26B and 27B. The total cell and non-nucleated cell increases in 26C were better than those in either 26A and 26B. However, in 27D they

were poorer than those of 27A and 27B. The nucleated cell decrease in 26C was less than that of the Amigen culture (26B) but more than that of the control culture (26A). In 27D it would appear that the opposite was true.

In 27C where liver extract (0.15 U.S.P. units per ml. of culture medium) was added alone the nucleated cell response was better than in any of those in 27A, 27B or 27D while the changes in the total cell concentration were roughly the same as those in 27A, 27B and 27D and the non-nucleated cell decrease more.

These results show that in the presence of liver extract the deleterious effect of Amigen is less marked than when Amigen alone is added. The combined influence of Amigen and liver extract produces a growth which does not appear to be significantly better than the fortification resulting from the use of added serum alone, and probably is not as good. In other words, the presence of synthetic amino acids appears to act adversely on the culture, and while this influence is lessened by the presence of liver extract, the use of Amigen appeared to be contraindicated.

F. Effect of Homologous Serum and Liver Extract

Since liver extract had a beneficial effect when added to Osgood's fluid alone the possibility presented itself that it might augment growth in cultures

where the culture medium contained homologous serum. Figures XV and XVII illustrate experiments in which this possibility was tested.

In 29B and 29D 0.15 U.S.P. units of liver extract per ml. of culture medium were added to the serum-fortified Gey's medium. In 29B the increase in non-nucleated and total cells while present was noticeably less than that in the controls 29A and 29C (described above in C) but in 29D the increase was approximately the same as that in 29A and 29B. On the other hand the decrease in nucleated cells was less in 29B and about the same in 29D as that in the controls.

The concentration of liver extract in 31A and 31B was 0.0015 U.S.P. units per ml. of culture medium. The response of all the cells in 31B was similar to those of the controls (31C and 31D which are described above in C). In 31A the response of the nucleated cells was much better, of the non-nucleated cells poorer and of the total cells about the same as those of the controls.

The results indicated that in the concentrations used liver extract has a slight deleterious or no effect on bone marrow cells cultured in serum-fortified Gey's medium.

G. Effect of Foreign Serum and Liver Extract

While operating on rabbit 30 insufficient blood was aspirated from which to obtain homologous serum to reinforce the culture medium in which the bone marrow cells from this rabbit were to be cultured. There was on hand serum from another rabbit killed the day before and this was used in this experiment instead of homologous serum. The culture medium therefore consisted of 67% Gey's fluid and 33% foreign serum. 0.0015 U.S.P. units of liver extract per ml. of culture medium were added to 30C and 30D. Figure XVI illustrates these results.

There was a good increase in the non-nucleated cells in 30B and a moderately good increase in 30A. The total cells in 30A fell off slightly and in 30B they remained at an almost constant level. The nucleated cells dropped off in number in both 30A and 30B but slightly more in 30A.

The liver extract cultures 30C and 30D showed somewhat better responses in all the cells than those of the controls (30A and 30B) with perhaps the exception of the nucleated cells in 30C which decreased about the same as those in 30B.

The indications are that liver extract added to Gey's fluid fortified with foreign serum increases the erythropoiesis to a slight degree. Gey's fluid fortified with foreign serum appeared to give a good

increase of red cells in the one experiment of this type.

H. Effect of Human Serum

Figures XVIII and XX show the effect of replacing the homologous serum in the culture medium with human serum. The cultures 32A, 32C, 34C and 34D are described above in C. Cultures 32B and 32D were cultured in 67% Gey's fluid and 33% human serum from C.L.N. 34A and 34B were cultured in human serum from W.J.M.

The initial absolute numbers of total cells in 32B and 32D were less than those of the controls and the absolute numbers of the non-nucleated cells were also moderately less than those of the controls. As shown in Figure XVIII the nucleated cells in both cultures dropped almost precipitously from the very start of the culture. The numbers of total cells in these cultures also dropped off markedly especially in 32D. The non-nucleated cells however in the two cultures do not seem to behave in the same way with respect to one another. They decreased very markedly in 32D but in 32B they increased very noticeably above the 100% level between the second and fourth hours after which they dropped off to slightly below the 100% level at the end of 6 hours.

In 34A and 34B the absolute number of total cells and non-nucleated cells at the zero hour was very

much less than those of the controls (34C and 34D). There were hardly any red cells in these two cultures at the beginning of the culture period. The nucleated cells were of about the same concentration at zero hours in the four cultures shown in Figure XX. The nucleated cells fell off markedly in 34A and 34B as they did in 32B and 32D. The total cells also fell off in number in 34A and 34B but showed terminal rises. There was a very noticeable increase in the numbers of non-nucleated cells in both 34A and 34 B. These are not shown in the graphs since the initial concentrations were so low that it was thought that expressing the growth in relative terms in this case would give a false picture.

The appearance of clumps of cells in all the cultures containing human serum as well as the development of a "clarity" in culture medium was noted after a short time of incubation in these cultures.

It is concluded that the human serum agglutinated and hemolyzed the rabbit bone marrow and red cells to a variable extent even before the cultures were incubated. However it would appear from 34 A and 34B that red cells were being produced after the initial agglutination and hemolysis had taken place or that they were being produced faster than they were being agglutinated or hemolyzed. In other words while on one hand the human serum was agglutinating and hemolyzing the cells it was supplying nutrient materials on the other hand for

the production of new red cells, and reducing the mature erythrocyte population. The significance of the latter will be discussed later.

I. Effect of Vitamin B₁₂

Figures XIX, XX and XXI show the effects of a culture medium of 67% Gey's solution and 33% homologous serum reinforced with 0.0015 mgm. of Vitamin B₁₂ per ml. of culture medium as compared to control cultures in 67% Gey's fluid and 33% homologous serum (described above in C).

The responses of the non-nucleated cells and total cells of 33C and 33 D were very similar to one another and the increases in them were of a degree lying between those observed in the control cultures. In other words one of the controls showed a better increase and the other a poorer increase in the number of total and non-nucleated cells than did the cultures 33C and 33D (containing Vitamin B₁₂). The nucleated cell decreases were very similar in the four cultures.

Cultures 34E and 34F (containing Vitamin B₁₂) showed decreases (with a slight increase at 4 hours) in all the cells comparable to the control culture 34D. The responses in total and non-nucleated cells in 34C was much better than that of those in 34E and 34F as well as those of control culture 34D.

The variability between the respective

control cultures does not allow any conclusions to be drawn with respect to the effect of Vitamin B₁₂ in the concentrations used.

J. Effect of Siliconed Flasks

Figure XXII shows the effects of culturing bone marrow cells in siliconed flasks.

The non-nucleated and total cell increases were very similar to those of the controls 35A and 35B (described above in C). The nucleated cells however did not fall off quite as much in the cultures in siliconed flasks as did those of the controls.

Indications are that siliconed flasks maintain the nucleated cell concentration at a higher level but that this effect is not marked.

K. Results of Rat Bone Marrow Cultures

The results of the rat bone marrow cultures are given in Appendix II. Of the seven cultures slight increases in non-nucleated cell counts were obtained in only three. The nucleated and total cell counts decreased in nearly all the cultures. Difficulty was encountered in obtaining sufficient blood from the rats from which serum could be obtained.

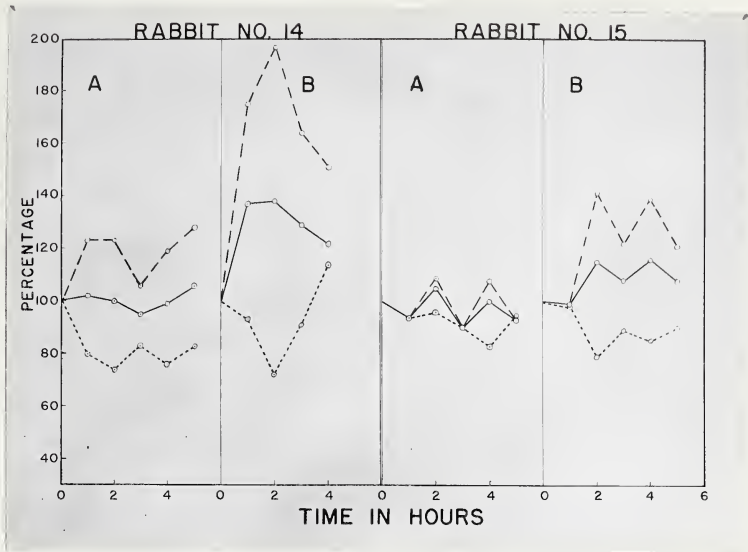
These results showing unsatisfactory growth in the cultures indicate that the technique of the method as developed for culturing rabbit bone marrow is perhaps not satisfactory for rat bone marrow.

LEGEND FOR FIGURES III - XXIII

———— Total Cells
..... Nucleated Cells
- - - - Non-nucleated Cells

Figure III

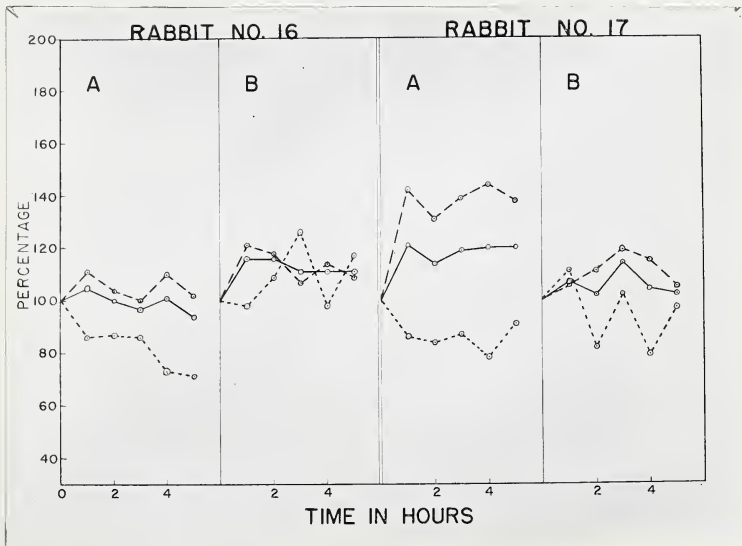
Growth Curves of Cultures from Rabbits No. 14 and 15



Cultured in 67% Osgood's fluid and 33% homologous serum.

Figure IV

Growth Curves of Cultures from Rabbits No. 16 and 17

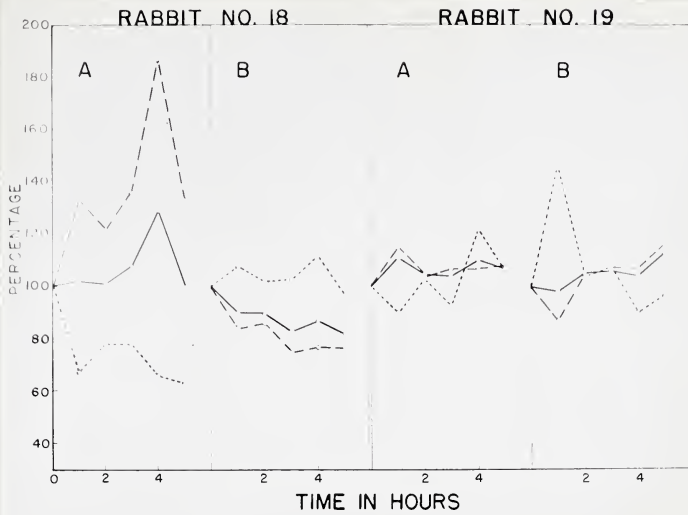


16A and 16B cultured in 90% Osgood's fluid and 10% homologous serum.

17A and 17B cultured in 67% Osgood's fluid and 33% homologous serum.

Figure V

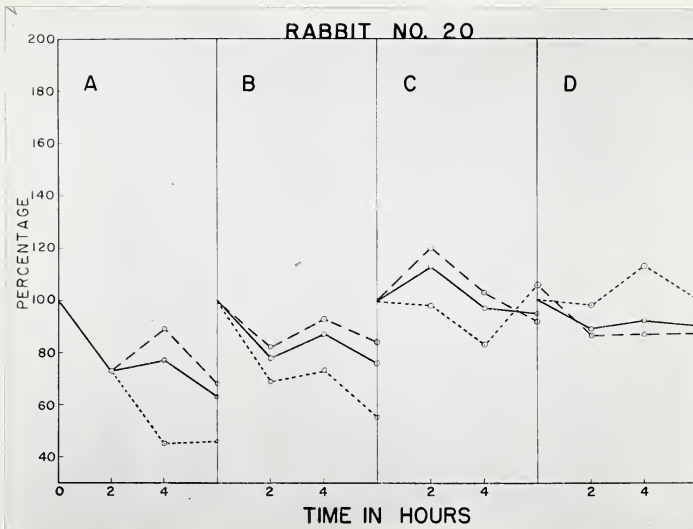
Growth Curves of Cultures from Rabbits No. 18 and 19



Cultured in 67% Osgood's fluid and 33% homologous serum.

Figure VI

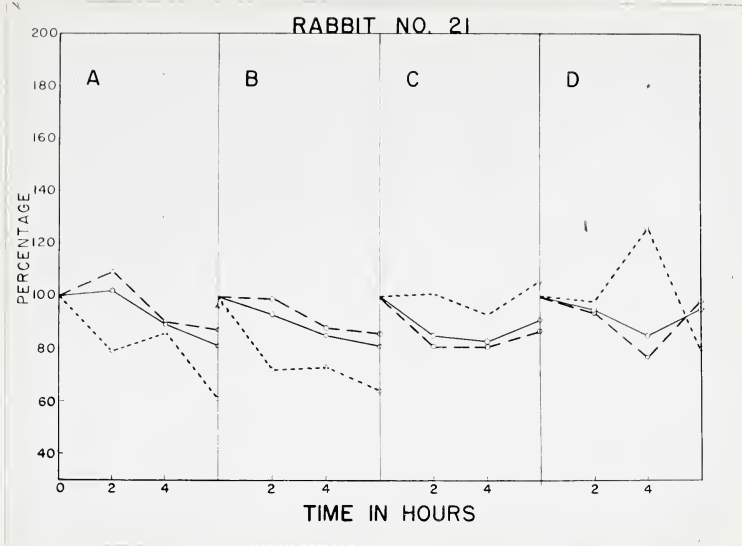
Growth Curves of Cultures from Rabbit No. 20



20A and 20B cultured in Osgood's fluid.
 20C and 20D cultured in Osgood's fluid fortified with 0.067 U.S.P. units of liver extract per cc. of culture fluid.

Figure VII

Growth Curves of Cultures from Rabbit No. 21

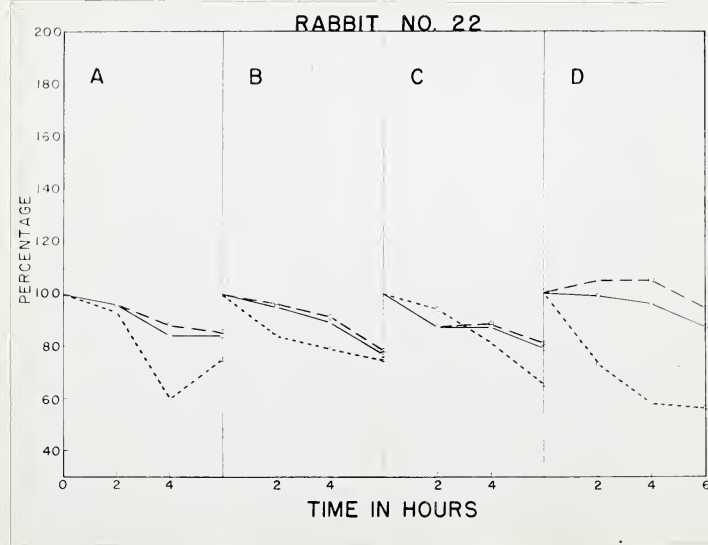


21 A and 21 B cultured in Osgood's fluid.

21 C and 21 D cultured in Osgood's fluid fortified with 0.67 U.S.P. units of liver extract per cc. of culture fluid.

Figure VIII

Growth Curves of Cultures from Rabbit No. 22

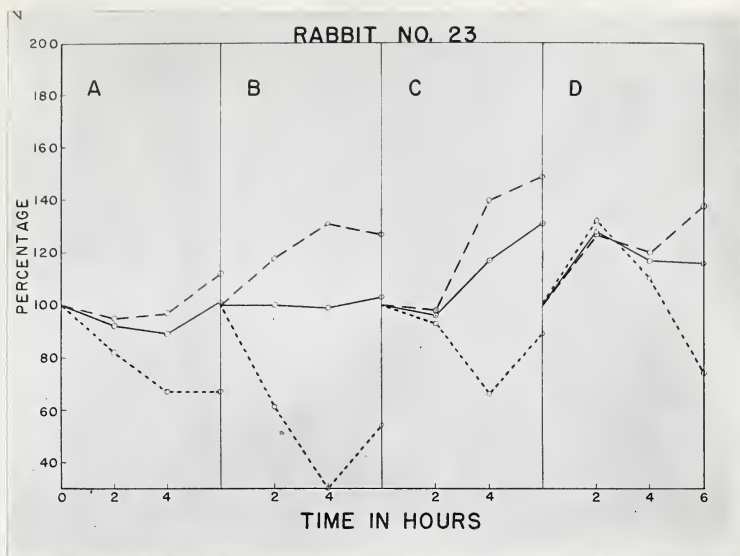


22A and 22 B cultured in Osgood's fluid.

22C and 22 D cultured in Osgood's fluid fortified with 0.0067 U.S.P. units of liver extract per cc. of culture fluid.

Figure IX

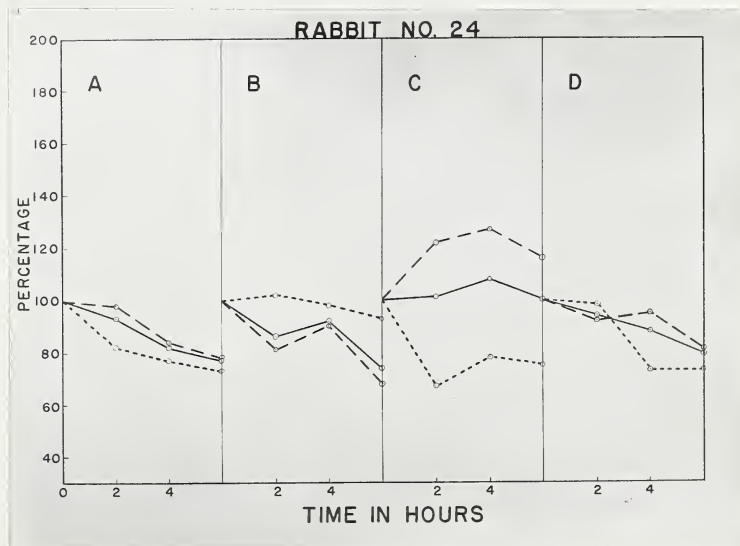
Growth Curves of Cultures from Rabbit No. 23



23 A and 23 B cultured in Osgood's fluid.
 23 C and 23 D cultured in Osgood's fluid fortified with
 0.15 U.S.P. units of liver extract per cc. of culture
 fluid.

Figure X

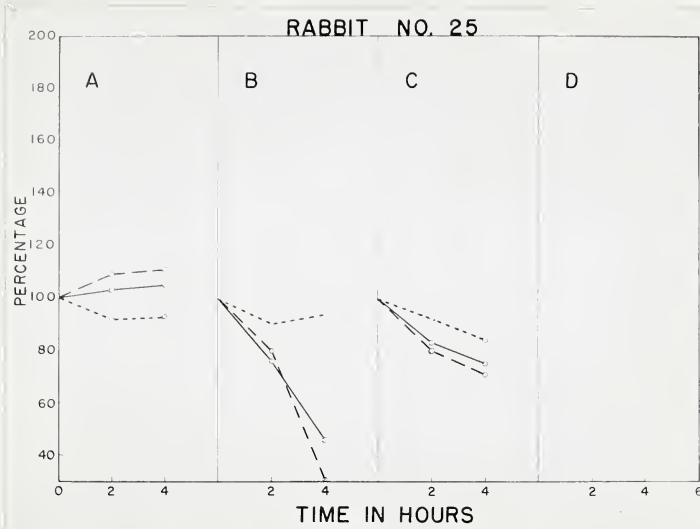
Growth Curves of Cultures from Rabbit No. 24



24 A and 24 B cultured in Osgood's fluid.
 24 C and 24 D cultured in Osgood's fluid fortified with
 0.15 U.S.P. units of liver extract per cc. of
 culture fluid.

Figure XI

Growth Curves of Cultures from Rabbit No. 25



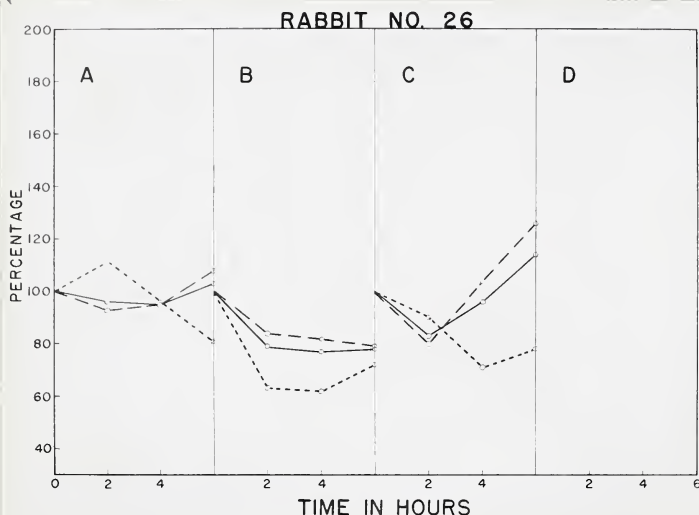
25A cultured in 67% Osgood's fluid and 33% homologous serum.

25B cultured in Osgood's fluid fortified with 0.067 cc. Amigen per cc. of culture fluid.

25C cultured in Osgood's fluid fortified with 0.067 cc. Amigen and 0.15 U.S.P. units of liver extract per cc. of culture fluid.

Figure XII

Growth Curves of Cultures from Rabbit No. 26



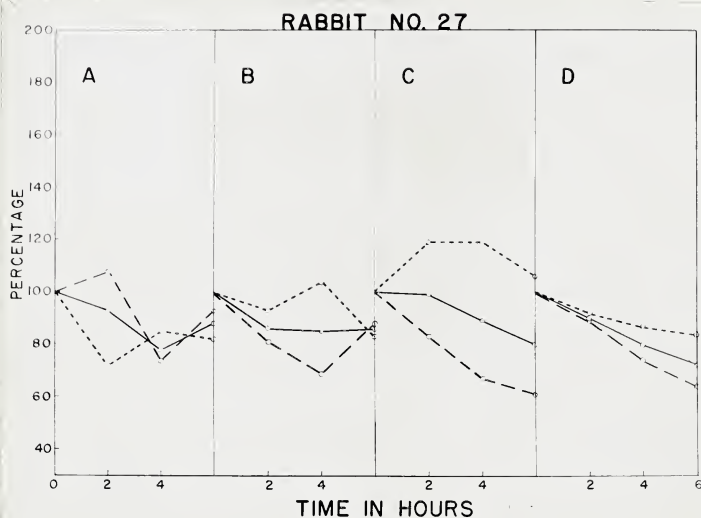
26A cultured in 67% Osgood's fluid and 33% homologous serum.

26B cultured in Osgood's fluid fortified with 0.01 cc. Amigen per cc. of culture fluid.

26C cultured in Osgood's fluid fortified with 0.01 cc. Amigen and 0.15 U.S.P. units of liver extract per cc. of culture medium.

Figure XIII

Growth Curves of Cultures from Rabbit No. 27



27A cultured in 67% Osgood's fluid and 33% homologous serum.

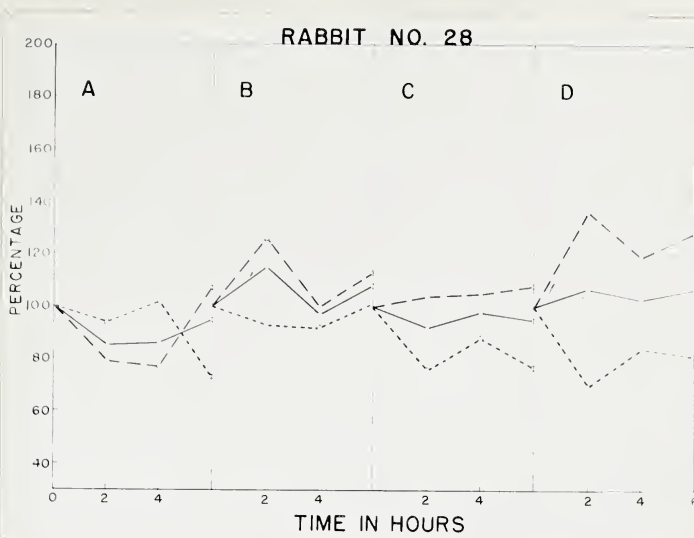
27B cultured in Osgood's fluid fortified with 0.01 cc. Amigen per cc. of culture fluid.

27C cultured in Osgood's fluid fortified with 0.15 U.S.P. units of liver extract per cc. of culture fluid.

27D cultured in Osgood's fluid fortified with 0.01 cc. Amigen and 0.15 U.S.P. units of liver extract per cc. of culture medium.

Figure XIV

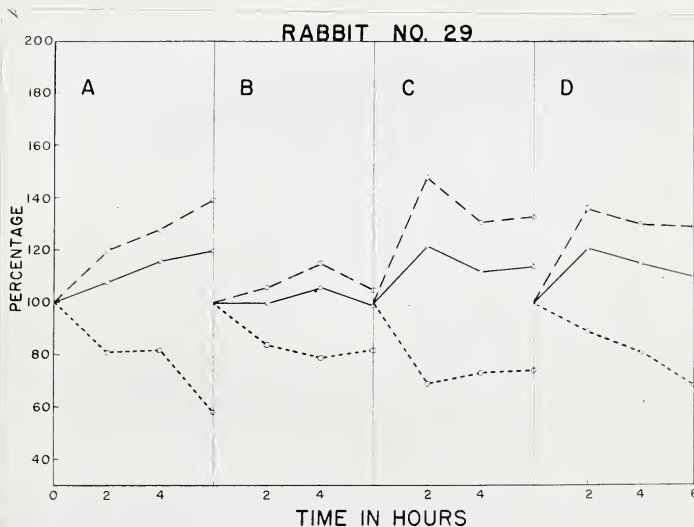
Growth Curves of Cultures from Rabbit No. 28



28A, 28B, 28C, and 28D cultured in 67% Gey's fluid (suitable for work under atmospheric conditions) and 33% homologous serum.

Figure XV

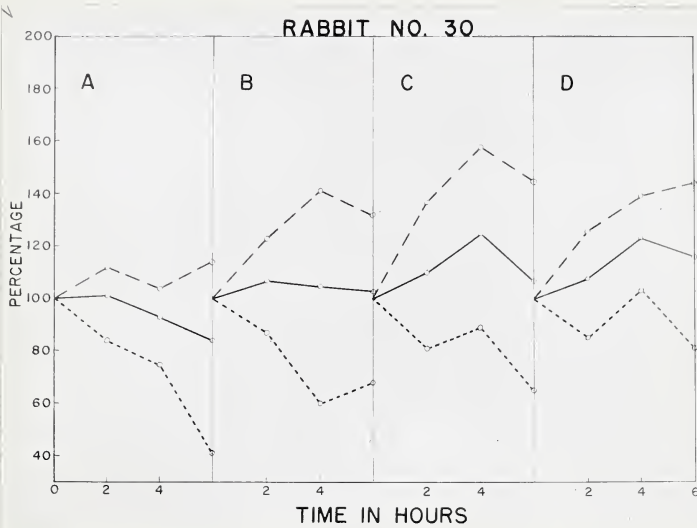
Growth Curves of Cultures from Rabbit No. 29



29A and 29C cultured in 67% Gey's fluid and 33% homologous serum.
 29B and 29D cultured in 67% Gey's fluid and 33% homologous serum fortified with 0.15 U.S.P. units of liver extract per cc. of culture medium.

Figure XVI

Growth Curves of Cultures from Rabbit No. 30

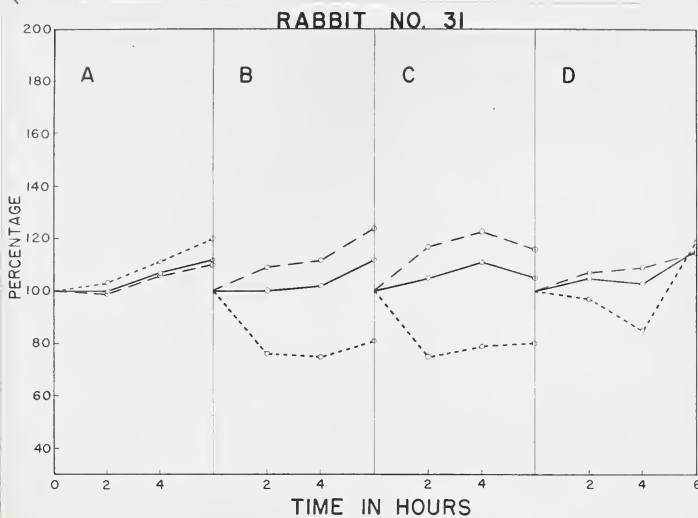


30A and 30B are cultured in 67% Gey's fluid and 33% foreign serum.

30C and 30D are cultured in 67% Gey's fluid and 33% foreign serum fortified with 0.0015 U.S.P. units of liver extract per cc. of culture medium.

Figure XVII

Growth Curves of Cultures from Rabbit No. 31

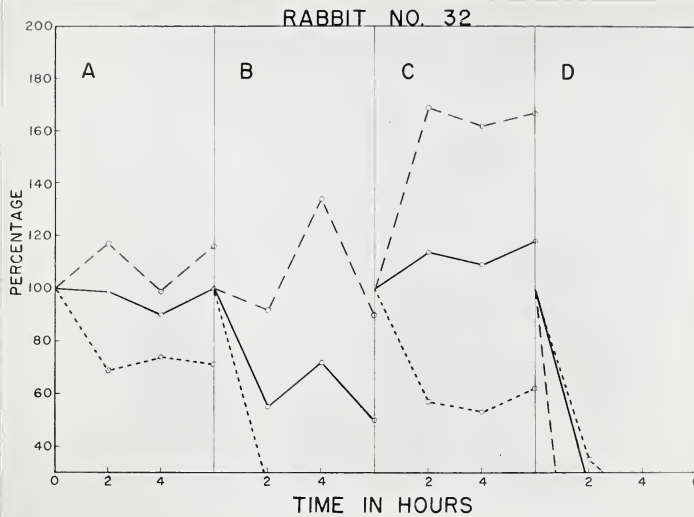


31C and 31D are cultured in 67% Gey's fluid and 33% homologous serum.

31A and 31B are cultured in 67% Gey's fluid and 33% homologous serum fortified with 0.0015 U.S.P. units of liver extract per cc. of culture medium.

Figure XVIII

Growth Curves of Cultures from Rabbit No. 32

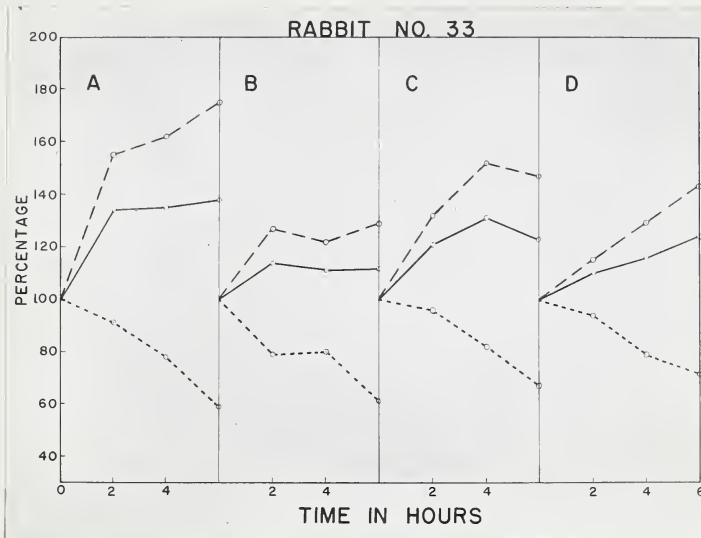


32A and 32C cultured in 67% Gey's fluid and 33% homologous serum.

32B and 32D cultured in 67% Gey's fluid and 33% human serum (from C.L.N.).

Figure XIX

Growth Curves of Cultures from Rabbit No. 33



33A and 33B cultured in 67% Gey's fluid and 33% homologous serum.
 33C and 33D cultured in 67% Gey's fluid and 33% homologous serum fortified with 0.0015 mgm. of Vitamin B₁₂ per cc. of culture medium.

XII. 1918

1. The first of the year was a very cold day.

2. The second day was a very cold day.

3. The third day was a very cold day.

4. The fourth day was a very cold day.

5. The fifth day was a very cold day.

6. The sixth day was a very cold day.

7. The seventh day was a very cold day.

8. The eighth day was a very cold day.

9. The ninth day was a very cold day.

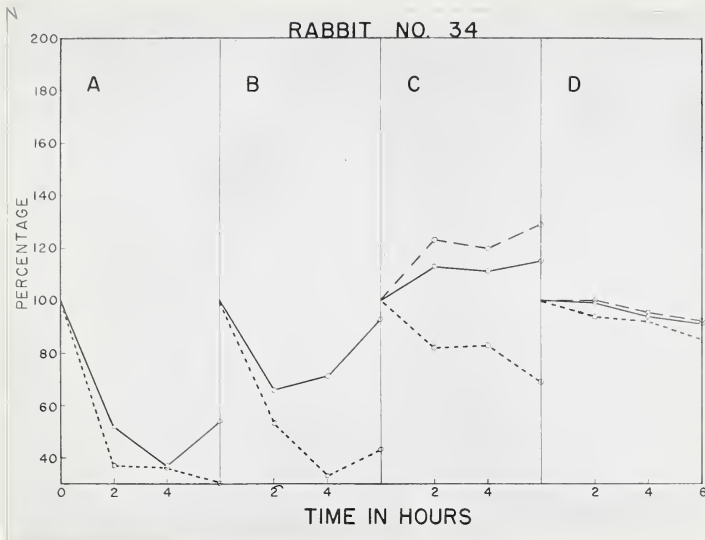
10. The tenth day was a very cold day.

11. The eleventh day was a very cold day.

12. The twelfth day was a very cold day.

Figure XX

Growth Curves of Cultures from Rabbit No. 34

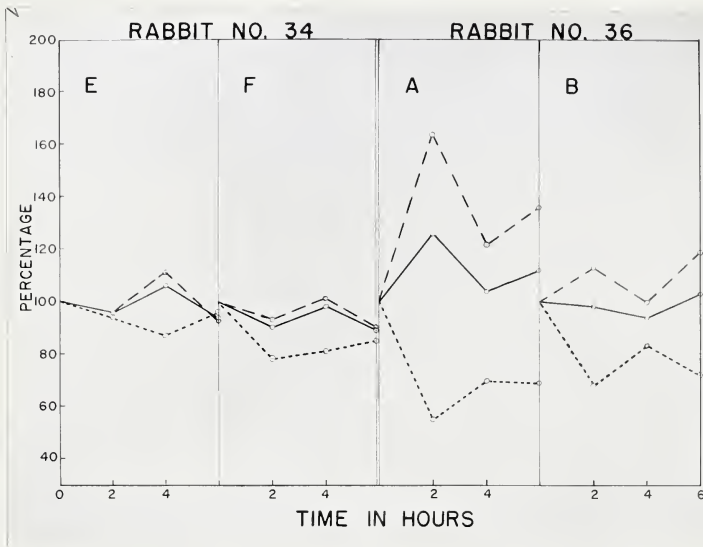


34A and 34B cultured in 67% Gey's fluid and 33% human serum (from W.J.M.).

34C and 34D cultured in 67% Gey's fluid and 33% homologous serum.

Figure XXI

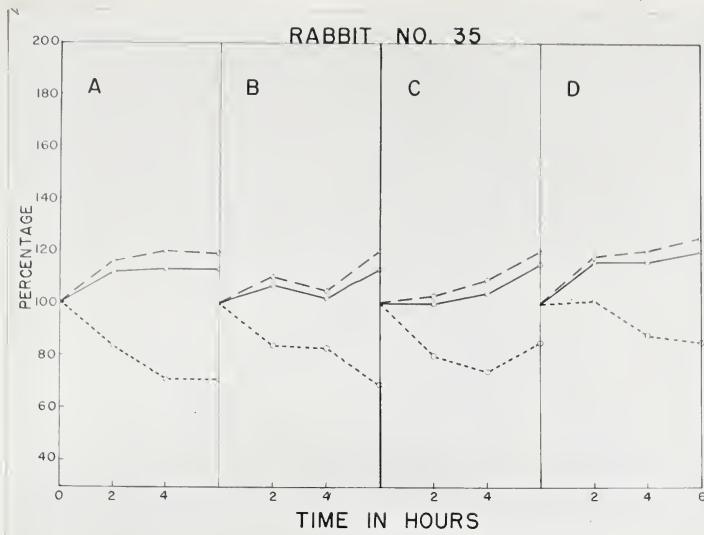
Growth Curves of Cultures from Rabbits No. 34 and 36



34E and 34F cultured in 67% Gey's fluid and 33% homologous serum reinforced with 0.0015 mgm. of Vitamin B₁₂ per cc. of culture medium.
36A and 36B cultured in 67% Gey's fluid and 33% homologous serum.

Figure XXII

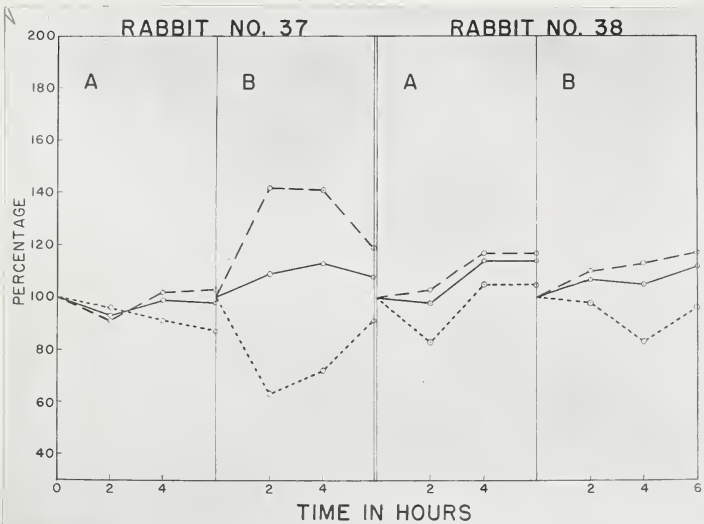
Growth Curves of Cultures from Rabbit No. 35



35A, 35B, 35C and 35D cultured in 67% Gey's fluid and 33% homologous serum.
35C and 35D cultured in siliconed flasks.

Figure XXIII

Growth Curves of Cultures from Rabbits No. 37 and 38



37A, 37B, 38A and 38B cultured in 67% Gey's fluid and 33% homologous serum.

SECTION VII

DISCUSSION OF RESULTS AND CONCLUSIONS

Preliminary work on this project on the accuracy of red cell counts indicated that there was a possible error in these counts (i.e., the average of the counts of two chamberfuls) of about 10%. The possible error in the nucleated cell count is probably higher because of the difficulty in determining whether some "cells" are actual nucleated cells or just debris. The possible error in the non-nucleated cell count is the largest because it is calculated from the above two and may therefore show the combined error. However, trends

reinforce the accuracy of previous counts so that if there is a persistent upward or downward trend in the cell counts over the culture period a fairly good degree of accuracy in the counts is indicated.

In the early work on counting technique an attempt was made to express the results of counting in statistical terms. Successive counts made on a given suspension showed a coefficient of variation of 4.5% for all errors. Since duplicate counts were made on each culture ~~it~~ seems unlikely that an error exceeding 10% would occur. Further, as already noted the trend of a culture based on serial counts at intervals, reinforces the figures for a culture at any one time. Such trends are in the main evident upon inspection of the graphs.

Consideration of the accuracy of counting methods is of course crucial in the scientific evaluation of the results. This is especially so where marked or obvious cell growth did not occur. It is however not a purpose of this thesis to decide the limits of accuracy of the counting methods, but rather to lay the groundwork for developing a method of culture which would be so positive as to leave no doubt when growth did occur. The thesis thus deals mainly with the factors which might be important in assuring the greatest growth using the simplest method.

A. Duration of Culture Period

The results of twelve cultures in the early part of the project (discussed in part B of Results) indicated that the major part of red cell production in vitro took place in the first few hours (approximately 6-8 hours). There are several explanations as to why this initial production of red cells was not maintained. Some or all of the nutrient materials in the culture medium may have been exhausted in this time, or the stimulus for red cell production (Plum, 104) may not have persisted. The by-products of cellular metabolism may have altered the medium with respect to pH and thus interfered with erythropoiesis. With the production of new red cells the mounting concentration of red cells may have been a factor itself in slowing the erythropoiesis. This point is discussed later. A certain amount of hemolysis may have taken place with the liberation of free hemoglobin which has been thought by some to interfere with erythropoiesis. The conditions of the culture may have been more suited to the production of red cells from normoblasts than the production of normoblasts from immature erythroid cells. In this case when the initial generation of normoblasts had been exhausted erythropoiesis would have to be slowed down to correspond to the pace at which normoblasts could be produced. The possibility of bacterial contamination with the production of toxins which may depress erythropoiesis must not be lost sight

of. However gross bacterial contamination was observed only in one culture in the preliminary series the results of which were not reported and this reason is not regarded as being of much significance.

B. Decrease of Nucleated Cells

In the majority of the cultures the nucleated cells decreased. Since this decrease was always marked when there was a marked production of red cells (although there were times when they decreased in number when red cell production was not marked) it is believed that many of the nucleated cells were used up in the formation of red cells and that for some reason the multiplication of the nucleated cells did not keep pace with their utilization in erythropoiesis.

Cairns and Lajtha (23) noticed a decrease in the white cell population in their cultures which they showed was due to adhesion of the white cells to the surface of the glass culture flask. Since these are nucleated cells such a phenomenon could assist in explaining the nucleated cell count drop. Cairns and Lajtha overcame this difficulty by using siliconed flasks, to the surfaces of which white cells did not adhere. Results shown in Figure XXII indicate that siliconed flasks are of some assistance in maintaining the nucleated cell count but that they are not the complete answer.

Leucocytes do not have a very long life span (especially the polymorphs) and their mortality during the culture period would also assist in lowering the nucleated cell count. Since differential cell counts were not made on the smears of the cultures taken at various intervals the importance of this phenomenon cannot be evaluated accurately.

C. Density of Red Cells as a Factor in Limiting Erythropoiesis.

There must be some mechanism in the body which normally keeps the bone marrow from producing more cells than are necessary to maintain a peripheral concentration of about 5,000,000 red blood cells per cubic millimeter. The possibility of such a mechanism operating in vitro has already been referred to in A above in that it may explain why erythropoiesis was not maintained at the initial rate for a longer period. The data were examined with this end in view and it was seen that even in these cultures where the initial concentration of red blood cells was high there was often a good erythropoietic response. However it is extremely difficult to assess all the factors at work in these cultures. It may be that, in those cultures where the initial red cell concentration was high, a

large percentage of the myeloid-erythroid cells were erythroid and that this explains the good erythropoietic response in the face of a high red cell concentration. Differential counts were not made on the myeloid-erythroid cells to establish this explanation.

However further work has been carried on which is not reported in this thesis but which was guided by the above concepts. Results thus far indicate the use of a phytoagglutinin obtained from crude extracts of beans has permitted the development of cultures showing more consistent and higher rates of growth than those reported in this thesis. The possibility that this more recent improvement in technique is due to the preferential reduction by agglutination of mature erythrocyte population exists. It is also possible that the residue of bean extract in the solution after agglutination has occurred may provide some beneficial nutrient to the cultures.

D. Osgood's and Gey's Fluids Compared

The amount of red cell formation obtained with Gey's fluid fortified with homologous serum was more consistent than that obtained with Osgood's fluid fortified with homologous serum. Consistency of results is exceedingly important because unless control cultures

have a fair degree of consistency between them it is difficult to draw any conclusions concerning the test cultures. The major difference between Osgood's and Gey's fluids is the presence of the bicarbonate ion in Gey's fluid. Cameron (24) has stated that the bicarbonate ion is necessary in any culture medium because it is important in the glycolytic activity of the cells. The bicarbonate ion is also of use in buffering pH changes in the medium. For these reasons Gey's fluid would appear to be superior to Osgood's fluid in bone marrow cultures as a culture medium.

E. Homologous Serum as a Nutrient

The growth response in cultures containing homologous serum in the culture medium was in most cases very much better than in those where the culture medium was Osgood's fluid alone. The results obtained with serum-fortified culture medium were also better on the whole than those obtained using media fortified with liver extract, Amigen or human serum. The conclusion reached is that homologous serum is a better nutrient fluid than any of the above. This is in accordance with Plum's results (104) from which he concluded that native (homologous) serum gave the best results.

The question arises of variability between sera of different rabbits being the cause of the variation in red cell production in cultures of bone marrow from different rabbits. If pooled serum was used these variations in response may be reduced. In this case however the problem of storing the pooled serum for a period of time without deterioration would arise. Plum (104) found that "foreign"* serum (and pooled serum would be similar) gave on the average 15% less growth than cultures containing the same amount of native serum (homologous). This extra 15% production of red cells would presumably be lost by using pooled serum. There was not time to run a series of experiments to check this point. In the one rabbit in which foreign serum was used there was growth comparable to that obtained with homologous serum. Because homologous serum was readily available from the rabbit supplying the culture, and also because of Plum's reported findings, homologous serum was used.

F. Foreign Serum as a Nutrient

No conclusions can be drawn from the results concerning the relative merits of foreign and native or homologous serum. As stated above Plum (104) found that

* That is serum from an animal other than that which provided the marrow cells.

native serum gave a 15% better response in erythropoiesis than did foreign serum. It is noted however that the foreign serum and added liver extract gave a better response than foreign serum alone. Since liver extract did not improve the growth obtained with homologous serum it is possible that when added to foreign serum it supplies whatever is lacking in foreign serum and is present in homologous serum to give a better response. More data would be necessary in order to clarify this point.

G. Human Serum as a Nutrient

The agglutination and hemolysis of the rabbit red blood cells when they are cultured in a medium containing human serum is a serious disadvantage in using human serum as a nutrient fluid for these cultures. However, Cairns and Lajtha (23) found that heating serum to 56° C. for an hour inactivated the agglutinins. If this procedure proves to be effective in removing the agglutinins human serum may be used to fortify the medium for the culture of rabbit marrow. Large amounts of serum could then be prepared with relative ease.

Further attempts to use human serum may permit the direct comparison of erythropoiesis resulting

from serum of normal and cancerous patients. This is the ultimate aim of this project. The investigation of methods to eliminate the agglutinins present in human sera are therefore important.

H. Effects of Liver Extract, Amigen and Vitamin B₁₂

The beneficial effect of liver extract in increasing erythropoiesis in vitro is in agreement with Plum's results (104). It is noteworthy that this beneficial effect was not manifest when liver extract and homologous serum were combined to fortify the culture medium indicating that the substances present in liver extract which increase erythropoiesis are present in homologous serum.

A series of experiments was performed in an attempt to provide the amino acids which are presumably necessary in the production of red cells. These amino acids were provided in the form of Amigen. The results from this series showed that Amigen had a deleterious effect on the bone marrow cells in the concentrations used. Perhaps amino acids provided in some other form would give better results.

It is interesting to note that the deleterious effect of Amigen was largely overcome by liver extract.

The information concerning the effect of Vitamin B₁₂ was too limited to draw any definite conclusions. However clinical experience indicates that Vitamin B₁₂ has a similar effect on erythropoiesis to that of liver extract. Since the effect of Vitamin B₁₂ in the above experiments was observed in a medium already fortified with homologous serum it is possible that a beneficial effect of the B₁₂ was masked by the known beneficial effect of the serum.

Early in this work the decision of whether to fortify the media with liver extract or with Vitamin B₁₂ had to be made. It was concluded that since B₁₂ was relatively new at the time it would be safer to use liver extract, the "in vivo" erythropoietic responses of which were well known.

I. Conclusions

1. It would appear that a simple and short-time method has been developed for the "in vitro" culture of bone marrow in which erythropoiesis can be studied.

2. The major erythropoietic response occurs in the first few hours permitting satisfactory observations over a six-hour period.

3. The most satisfactory erythropoietic response was obtained with a culture medium of Gey's or Osgood's fluids fortified with homologous serum.

4. Attempts to improve the growth above the levels obtained with added serum using liver extract, Amigen and Vitamin B₁₂ were unsuccessful.

5. It was in consequence impossible to employ a completely synthetic culture medium in which optimum growth could be obtained. The presence of serum appears to provide essential substances for maximum growth.

6. Leads for future experimentation were obtained.

SECTION VIII

SUMMARY

1. The incidence and causes of anemia in cancer have been reviewed.

2. The incidence and causes of anemia in tumor-bearing experimental animals have been reviewed and a possible association with a lowered liver catalase activity in these animals noted.

3. A review of a number of bone marrow culture methods has been presented.

4. A survey of the present concepts of erythropoiesis has been given.

5. The bone marrow culture methods were analyzed from the standpoint of certain criteria and that of Osgood and Brownlee chosen for this project.

6. A description of the apparatus and technique as employed in this method are given in detail.

7. Results of 101 bone marrow cultures are presented and conclusions drawn from them.

BIBLIOGRAPHY

1. Adams, D. H. The mechanism of the liver catalase depressing action of tumours in mice.
Brit. J. Cancer 4:183-95, 1950.
2. ----- Further observations on the liver catalase depressing action of tumours.
Ibid. 5:115-23, 1951.
3. Allbutt, T. C. A System of Medicine. 1905
The MacMillan Company. New York
Vol. III, p. 560.
4. Allbutt, T. C. and Rolleston, H. D. A System of Medicine. 1908. MacMillan and Co., Limited, St. Marten's Street, London.
Vol. III, p. 346.
5. Allibone, E. C. and Collins, D. H. Symtomatic haemolytic anaemia associated with ovarian teratoma in a child.
J. Clin. Path. 4:412-20, 1951.
6. Alvarez, W. C., Judd, E. S., MacCarty, W. C., and Zimmerman, A. R. The varying degrees of anemia produced by carcinoma in different parts of the colon.
Arch. Surg. 15:402-17, 1927.
7. Appleman, David; Skavinski, E. R., and Stein, A. M. Catalase studies on normal and cancerous rats.
Cancer Research 10:498-505, 1950.
8. ----- Catalase studies on protein-depleted rats bearing the Jensen sarcoma.
Cancer Research 11:926-9, 1951.
9. Ariel, Irving, Rekers, R. E., Pack, G. T., and Rhoads, C. P. Metabolic studies in patients with cancer of the gastro-intestinal tract: X. Hypoproteinemia and anemia in patients with gastric cancer.
Ann. Surg. 118:366-71, 1943.
10. Armburst, K. E. and Bett, H. D. Response of bone marrow explants to liver extract preparations.
J. Pharm. Exp. Ther. 88:382-7, 1946.

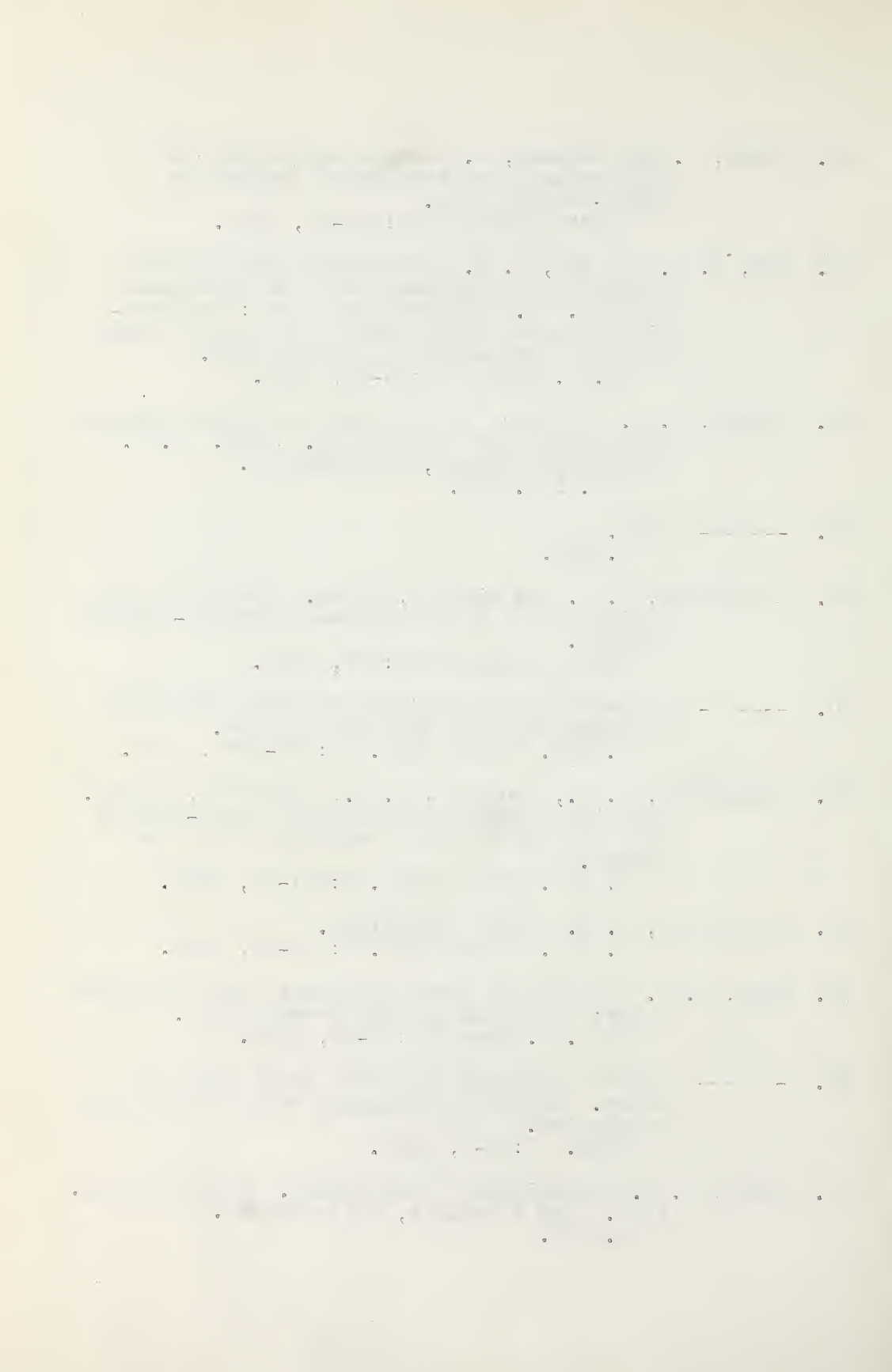
11. Armstrong, Margaret and Ham, Arthur. Effects, particularly anemia, produced in chicks by growth in their yolk sacs of mouse mammary tumors.
Cancer Research 7:481-90, 1947.
12. Bateman, J. C. A study of blood volume and anemia in cancer patients.
Blood 6:639-51, 1951.
13. Begg, R. W. Systemic effects of tumors in rats.
Cancer Research 11:341-4, 1951.
14. Begg, R. W. and Dickinson, T. E. Systemic effects of tumors in force-fed rats.
Cancer Research 11:409-12, 1951.
15. Berkson, Joseph; Magath, T. B., and Hurn, Margaret. Laboratory standards in relation to chance fluctuations of the erythrocyte count as estimated with the hemocytometer.
J. Am. Stat. Assoc. 30:414-26, 1935.
16. ----- The error of estimate of the blood cell count as made with the hemocytometer.
Am. J. Physiol. 128:309-23, 1940.
17. Biesele, J. J. and Berger, R. E. The effect of xanthopterin and related agents on the proliferation of rabbit marrow cells in vitro.
Cancer Research 10:686-93, 1950.
18. Blum, L. L. Photoelectric determination of erythrocyte count.
Am. J. Clin. Path., Vol. 15
Tech. Sect., 9:85-93, 1945.
19. Blumenthal, H. T. The effects of spontaneous and transplanted rat and mouse tumors on the red and white cells in circulating blood and bone marrow.
Cancer Research 1:196-204, 1941.
20. Brown, C. H., Colvert, J. R., and Brush, B. E. Clinical aspects of carcinoma of the cecum and ascending colon: Report of 60 cases.
Ann. Int. Med. 28:940-8, 1948.
21. Bryan, W. R., Chastain, L. L., and Garrey, W. E. Errors of routine analysis in the counting of leucocytes.
Am. J. Physiol. 113:416-29, 1935.

22. Bullowa, J. G. M., Osgood, E. E., Bukantz, S. C. and Brownlee, I. E. The effect of sulfa-pyridine alone and with serum on pneumococcic pneumonia and on pneumococcus-infected marrow cultures.
Am. J. M. Sc. 199:364-80, 1940.
23. Cairns, H. J. F., and Lajtha, L. G. Loss of white cells in bone marrow culture.
Nature 162:536-7, 1948.
24. Cameron, Gladys. Tissue Culture Technique. Second Edition, 1950. Academic Press Inc., Publishers, New York.
p. 37.
25. ----- Ibid.
p. 40.
26. Carrel, Alexis and Ebeling, A. H. Pure cultures of large mononuclear leucocytes.
J. Exper. Med. 36:365-78, 1922.
27. Cecil, R. L. A Textbook of Medicine by American Authors.
Sixth Edition, 1943. W. B. Saunders Company, Philadelphia and London.
p. 682.
28. ----- Ibid.
p. 745-6.
29. Clark, J. H., Nelson, W., Lyons, C., Mayerson, H. S., and DeCamp, P. Chronic shock: the problem of reduced blood volume in the chronically ill patient; concept of chronic shock; hemoglobin and red blood cell deficits in chronic shock; quantitative aspects of anemia associated with malignant tumors.
Ann. Surg. 125:618-46, 1947.
30. Clifton, E. E. and Wolstenholme, J. T. Hypervolemia and associated changes in mice bearing a transplanted granulosa cell tumor.
Cancer Research 9:331-5, 1949.
31. Commons, R. R. and Strauss, M. B. Myelophthisic anemia, the presenting manifestation of prostatic carcinoma with skeletal metastases; the effect of castration and stilbestrol.
Am. J. M. Sc. 215:525-9, 1948.

32. Connolly, V. J. A comparison of red blood cell counting technics.
Am. J. Clin. Path. 17:254-5, 1947.
33. Davis, J. E. Biochemical differences between mice of tumour and non-tumour strain, and tumour and non-tumour-bearing mice of tumour strain.
Can. Med. Assoc. J. 36:27-30, 1937.
34. Dickinson, T. E. and Begg, R. W. Liver catalase activity in rats bearing benign and malignant tumors.
Cancer Research 11:244, 1951.
35. Doan, C. A., Cunningham, R. S., and Sabin, F. R. Experimental studies on the origin and maturation of avian and mammalian red blood cells. 1925. Contributions to Embryology. Carnegie Institute of Washington.
Vol. 16; nos. 78-84; p. 163-226.
36. Dounce, A. L. and Shanewise, R. P. Liver catalase of tumor-bearing and leprous rats.
Cancer Research 10:103-7, 1950.
37. Downey, Hal. Handbook of Hematology. First Edition, 1938. Paul B. Hoeber Inc. Medical Book Department of Harper and Brothers. New York.
Vol. II, p. 1482-3.
38. Duran-Jorda, Frederic. Formation of red blood corpuscles.
Lancet 244:513-4, 1943.
39. ----- Formation of red blood corpuscles.
Ibid. 245:186-8, 1943.
40. ----- Secretion of red blood corpuscles.
Nature 159:293-4, 1947.
41. ----- The secretion of reticulocytes by the normoblast.
Acta Med. Scand. 136:275-82, 1950.
42. Eichhorst, Hermann. Handbook of Practical Medicine. 1886. William Wood and Company, New York.
Vol. II Diseases of the Digestive, Urinary and Sexual Apparatus. p. 80.
43. ----- Ibid.
p. 219.

44. Eisen, David. The anaemia of cancer of the gastro-intestinal tract. Based on a study of 187 cases.
Can. Med. Assoc. J. 17:307-13, 1927.
45. ----- Anaemia as a predominating symptom in malignant disease.
Ibid. 17:1506-9, 1927.
46. ----- Blood changes in malignant disease. An analysis of 353 cases.
Am. J. M. Sc. 176:200-10, 1928.
47. Emmel, V. E. The origin of erythrocytes by a process of constriction or budding.
Science 35:873-5, 1912.
48. ----- Concerning certain cytological characteristics of the erythroblasts in the pig embryo, and the origin of non-nucleated erythrocytes by a process of cytoplasmic constriction.
Am. J. Anat. 16:127-94, 1914.
49. ----- Studies on the non-nucleated elements of the blood. II. The occurrence and genesis of non-nucleated erythrocytes or erythroplastids in vertebrates other than mammals.
Am. J. Anat. 33:347-406, 1924.
50. Erdmann, Rhoda. Cytological observation on the behavior of chicken bone marrow in plasma medium.
Am. J. Anat. 22:73-126, 1917.
51. Ewing, James. Neoplastic Diseases. A treatise on tumors. Second Edition, 1922. W. B. Saunders Company, Philadelphia and London.
p. 67.
52. ----- Ibid.
p. 69.
53. Foot, N. C. The growth of chicken bone marrow in vitro and its bearing on hematogenesis in adult life.
J. Exper. Med. 17:43-60, 1913.
54. Furth, J. and Sobel, H. Hypervolemia secondary to grafted granulosa-cell tumor.
J. Nat. Cancer Inst. 7:103-13, 1946.

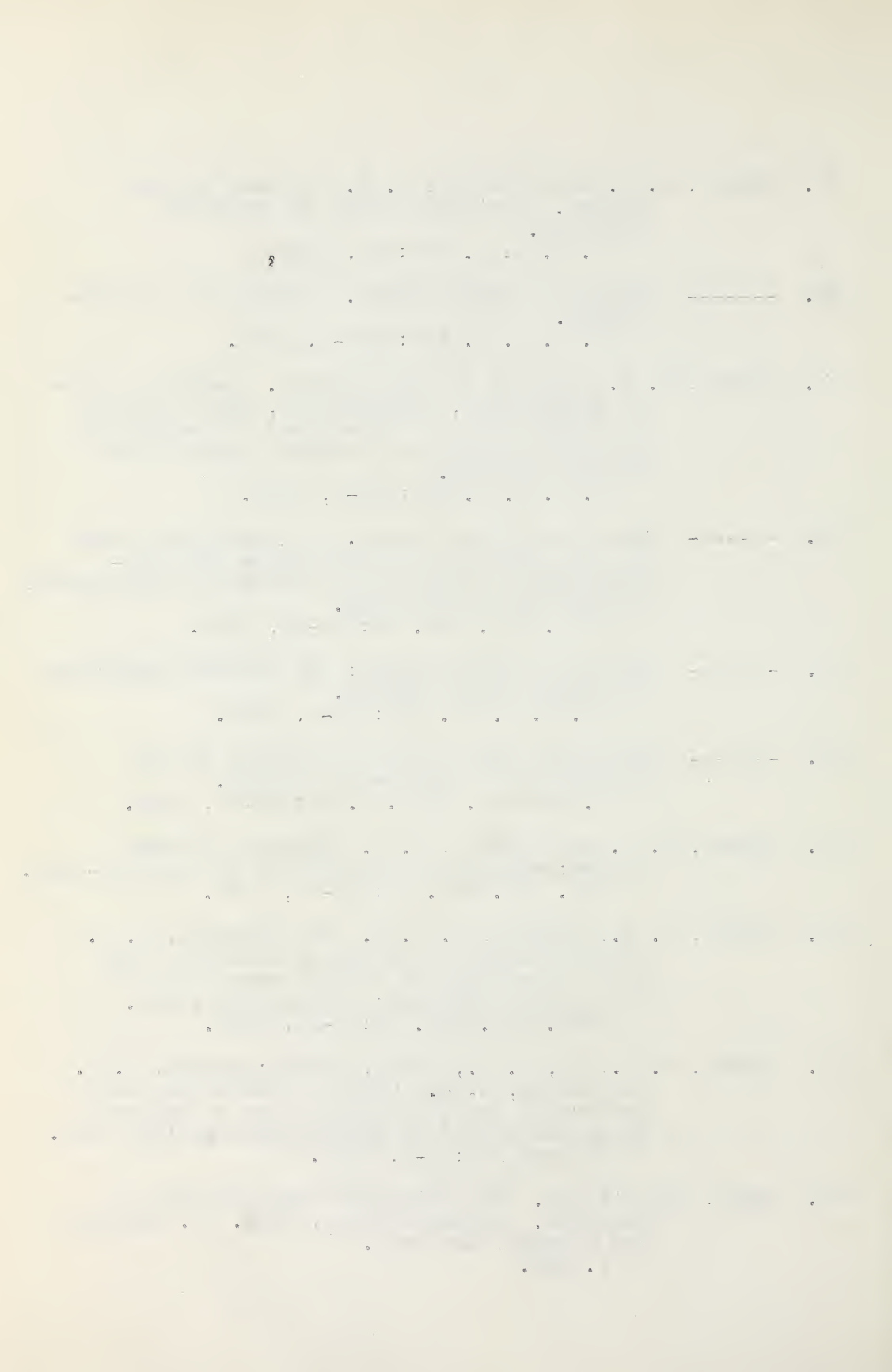
55. Furth, J. and Moshman, J. On the specificity of hypervolemia and congestive changes in tumor-bearing mice.
Cancer Research 11:543-51, 1951.
56. Gey, G. O. and Gey, M. K. The maintenance of human normal cells and tumor cells in continuous culture. I. Preliminary report: cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation.
Am. J. Cancer 27:45-76, 1936.
57. Gibson, G. A. Practice of Medicine by Eminent Medical Specialists and Authorities. 1901. J. P. Lippincott Company, Philadelphia.
Vol. I p. 695.
58. ----- Ibid.
p. 796.
59. Greenfield, R. E. and Meister, Alton. Studies of the inhibition of liver catalase in tumor-bearing animals.
Cancer Research 10:222, 1950.
60. ----- The effect of injections of tumor fractions on liver catalase activity of mice.
J. Nat. Cancer Inst. 11:997-1005, 1951.
61. Greenstein, J. P., Jenrette, W. V., and White, Julius. The liver catalase activity of tumor-bearing rats and the effect of extirpation of the tumors.
J. Nat. Cancer Inst. 2:283-91, 1941.
62. Greenstein, J. P. Tumor enzymology.
J. Nat. Cancer Inst. 3:419-47, 1943.
63. Gunz, F. W. Culture of human leukaemic blood cells in vitro; technique and the growth curve.
Brit. J. Cancer 2:29-41, 1948.
64. ----- Culture of human leukaemic blood cells in vitro. Normal and abnormal cell division and maturation.
Ibid. 2:41-8, 1948.
65. Haden, R. L. Principles of Hematology. Third Edition. 1946. Lea & Febiger, Philadelphia.
p. 226.



66. ----- Ibid.
p. 255.
67. ----- Ibid.
p. 269.
68. ----- Ibid.
p. 270.
69. Hays, E. E. Effect of folic acid upon primitive erythrocytes in vitro.
Proc. Soc. Exp. Biol. 63:558-60, 1946.
70. Isaacs, Raphael. Anemia in cancer.
Med. Clin. N. America 10:1219-33, 1927.
71. Israëls, M. C. G. The culture in vitro of leucocytes from human bone marrow.
J. Path. Bat. 50:145-51, 1940.
72. Klatt, O. A. and Taylor, Alfred. The effect of tumor growth on liver catalase concentration.
Cancer Research 11:764-7, 1951.
73. Le Gros Clark, W. E. Origin of erythrocytes.
Nature 159:579, 1947.
74. Lewis, M. R. The formation of macrophages, epithelioid cells and giant cells from leucocytes in incubated blood.
Am. J. Path. 1:91-100, 1925.
75. Lewis, M. R. and Lewis, W. H. Transformation of mononuclear blood-cells into macrophages, epithelioid cells, and giant cells in hanging-drop blood-cultures from lower vertebrates. 1926.
Contributions to Embryology. Carnegie Institute of Washington.
Vol. 18; nos. 90-7; p. 95-120.
76. Limarzi, L. R. Evaluation of bone marrow concentration techniques. A modified method for the simultaneous preparation and staining of blood and bone marrow films.
J. Laborat. Clin. M. 32:732-40, 1947.
77. Master, A. M. The blood count in carcinoma of the esophagus.
J. A. M. A. 84:734-6, 1925.

78. Mettier, S. R. Hematologic aspects of space consuming lesions of the bone marrow (myelophthisic anemia).
Ann. Int. Med. 14:436-48, 1940.
79. Morrison, Maurice. An analysis of blood picture in 100 cases of malignancy.
J. Laborat. Clin. M. 17:1071-93, 1932.
80. Norris, E. R. and Majnarich, J. J. Effect of xanthopterin on cell proliferation in bone marrow cultures.
Am. J. Physiol. 152:175-8, 1948.
81. ----- Further studies on xanthopterin and other pteridines in bone marrow cultures.
Ibid. 152:652-7, 1948.
82. ----- Effect of normal blood serum and blood serum from neoplastic disease on cell proliferation in bone marrow cultures.
Ibid. 153:483-7, 1948.
83. ----- Effect of pteridines and blood sera on human bone marrow cells in vitro.
Ibid. 153:496-8, 1948.
84. ----- Cell proliferation accelerating and inhibiting substances in normal and cancer blood and urine.
Proc. Soc. Exp. Biol. 70:229-34, 1949.
85. ----- Vitamin B₁₄ and cell proliferation.
Science 109:32-3, 1949.
86. ----- Action of enzymes on Vitamin B₁₄ and other pteridines.
Science 109:33-5, 1949.
87. Oppenheim, Abraham, Abels, J. C., Pack, G. T. and Rhoads, C. P. Metabolic studies in patients with cancer of gastro-intestinal tract: XIX. The anemia of patients with gastric carcinoma.
J. A. M. A. 127:273-6, 1945.
88. Osgood, E. E. and Muscovitz, A. N. Culture of human bone marrow. Preliminary report.
J. A. M. A. 106:1888-90, 1936.

89. Osgood, E. E. and Brownlee, I. E. Culture of bone marrow. A simple method for multiple cultures.
J. A. M. A. 107:123, 1936.
90. ----- Culture of human marrow. Details of a simple method.
J. A. M. A. 108:1793-6, 1937.
91. Osgood, E. E. Culture of human marrow. Length of life of neutrophils, eosinophils, and basophils of normal blood as determined by comparative cultures of blood and sternal marrow from healthy persons.
J. A. M. A. 109:933-7, 1937.
92. ----- Culture of human marrow. A comparative study of the effects of sulfanilamide and anti-pneumococcus serum on the course of experimental pneumococcic infections.
Arch. Int. Med. 62:181-98, 1938.
93. ----- Culture of human marrow: an improved apparatus for large scale culture.
Am. J. M. Sc. 195:141-4, 1938.
94. ----- Culture of human marrow as an aid in the evaluation of therapeutic agents.
J. Laborat. Clin. M. 24:954-62, 1939.
95. Osgood, E. E. and Bracher, G. J. Culture of human marrow; studies of the effects of Roentgen-rays.
Ann. Int. Med. 13:563-75, 1939.
96. Osgood, E. E., Bullowa, J. G. M., and Brownlee, I. E. Effectiveness of various sulfonamide drugs and neoarsphenamine against pneumococci in bone marrow cultures; comparative study.
Arch. Int. Med. 73:13-17, 1944.
97. Osgood, E. E., Li, J. G., Tivey, Harold; Duerst, M. L. and Seaman, A. J. Growth of human leukemic leucocytes in vitro and in vivo as measured by uptake of P32 in desoxyribose nucleic acid.
Science 114:95-8, 1951.
98. Osler, Sir William. The Principles and Practice of Medicine. Second Edition, 1895. D. Appleton and Company, New York.
p. 407.



99. Pearce, Louise and Casey, A. E. Studies in the blood cytology of the rabbit. VII. Observations on rabbits inoculated with a transplantable malignant neoplasm.
J. Exper. Med. 53:895-917, 1931.
100. Pierce, Mila. Cultures of leukemic blood leucocytes.
Arch. Path. 14:295-322, 1932.
101. ----- Cultivation of human leukemic leucocytes on the chorioallantoic membrane of the chicken egg.
Ibid. 34:538-45, 1942.
102. Pisciotto, A. V. Clinical and pathologic effects of space-occupying lesions of the bone marrow.
Am. J. Clin. Path. 20:915-33, 1950.
103. Plum, C. M. Methods for continuous tissue culture as applied to bone marrow.
Acta Physiol. Scand. 11:260-9, 1946.
104. ----- Some investigations of erythropoiesis in human bone-marrow. Cultivated in various media.
Acta Physiol. Scand. 14:383-98, 1947.
105. ----- In vitro study of bone marrow. I. A method for marrow culture.
Blood - the Journal of Hematology. Special Issue No. I, Morphologic Hematology. 1947, p. 33-41.
106. ----- In vitro study of bone marrow. II. Studies of erythropoiesis.
Ibid. p. 42-53, 1947.
107. ----- A new method for bone marrow culture.
Sang. 21:452-4, 1950.
108. ----- The production of erythrocytes.
Proceedings of the sixth international congress of experimental cytology.
109. Ponder, Eric. Hemolysis and Related Phenomena. 1948.
London. J. and A. Churchill Ltd., 104 Gloucester Place, W. I.
p. 354-6 The technic of red cell counting.

110. Rachmilewitz, M. and Rosin, A. Studies on bone marrow in vitro. I. The cellular pattern and behavior of explanted bone marrow.
Am. J. M. Sc. 206:17-26, 1943.
111. Rosin, A. and Rachmilewitz, M. Studies on bone marrow in vitro. III. The effect of anoxia and hyperoxia on explanted bone marrow.
Blood 3:165-74, 1948.
112. Rundles, R. W. and Jonsson, U. Metastases in bone marrow and myelophthisic anemia from carcinoma of the prostate.
Am. J. M. Sc. 218:241-50, 1949.
113. Rusznyák, St., Löwinger, S., and Lajtha, L. Maturation of megaloblasts in bone marrow cultures.
Nature 160:757-8, 1947.
114. Schwertman, A. J. Anemia of malignancy.
South. Med. J. 41:598-601, 1948.
115. Shen, S. C. and Homberger, Freddy. The anemia of cancer patients and its relation to metastases to the bone marrow.
J. Laborat. Clin. M. 37:182-98, 1951.
116. Singer, Karl and Dameshek, William. Symptomatic hemolytic anemia.
Ann. Int. Med. 15:544-63, 1941.
117. Skavinski, E. R. and Stein, A. M. The effect of tumor implants on chick embryo liver catalase activity.
Cancer Research 11:768-71, 1951.
118. Smith, C. Normal variations in erythrocyte and hemoglobin values in women.
Arch. Int. Med. 47:206-29, 1931.
119. Sobel, Harry and Furth, Jacob. Hypervolemia in mice bearing granulosa cell growths; time of onset and some associated physiological and chemical changes.
Endocrinology 42:436-47, 1948.
120. Stats, Daniel; Rosenthal, Nathan and Wasserman, L. R. Hemolytic anemia associated with malignant diseases.
Am. J. Clin. Path. 17:585-613, 1947.

121. Strong, L. C. Hemoglobin levels in various degrees of susceptibility to spontaneous tumors.
Am. J. Cancer 27:500-9, 1936.
122. Taylor, Alfred and Pollack, M. A. Hemoglobin level and tumor growth.
Cancer Research 2:223-7, 1942.
123. Taylor, D. R., McAfee, Marguerite and Taylor, Alfred. The effect of yolk sac-cultivated tumors on the hemoglobin level in the embryonic chick.
Cancer Research 3:542-5, 1943.
124. Thompson, W. P. and Illyne, C. A. The clinical and hematologic picture resulting from bone marrow replacement.
Med. Clin. N. America 24:841-53, 1940.
125. Totterman, L. E. Intravenous iron tolerance tests in malignant neoplasms. Their value for diagnosis and for antianemic treatment with iron, Vitamin C and penicillin.
Acta Med. Scand. 140:265-79, 1951.
126. Vaughan, J. M. Leuco-erythroblastic anaemia.
J. Path. Bact. 42:541-64, 1936.
127. ----- The Anaemias. Second Edition, 1936.
Oxford University Press, London: Humphrey Milford.
p. 159.
128. ----- Ibid.
p. 163.
129. Wajda, S. H. Origin of erythrocytes.
Nature 159:254-6, 1947.
130. ----- Origin of erythrocytes: reply to Le Gross Clark.
Ibid. 159:579, 1947.
131. Waugh, T. R. Hemolytic anemia in carcinomatosis of the bone marrow.
Am. J. M. Sc. 191:160-70, 1936.
132. Weisberger, A. S. and Heinle, W. R. Study of fixed tissue sections of sternal bone marrow obtained by needle aspiration. III. Metastatic carcinoma in sternal bone marrow.
Am. J. M. Sc. 217:263-8, 1949.

133. Wilson, F. H. Spring clips for "all glass" blood counting chambers.
Am. J. Clin. Path., Tech. Supp. 4:93-5, 1940.
134. Ying-Chang Ch'u and Forkner, C. E. Errors in erythrocyte counts due to Hayem's solution - avoided with Gower's solution.
J. Laborat. Clin. Med. 23:1282-93, 1938.

APPENDIX I.

i

Miscellaneous Expendable Material

1. Absorbant cotton. Johnson and Johnson.
2. Acetic acid 1%. Filtered to remove foreign material.
3. Acetone. Alberta National Drug Company.
4. Alcohol
 - a. 95% ethyl alcohol
 - b. 70% ethyl alcohol
 - c. Distilled 95% ethyl alcohol
 - d. Special acetone-free methyl alcohol for stains.
5. Amigen. Mead Johnson & Company. Amigen 5% in 5% dextrose solution. Aqueous solution of a pancreatic hydrolysate of casein containing amino-acids and small peptides.
6. Brewer thioglycolate medium. Difco Laboratories, Detroit, Michigan.
7. Calcium chloride. Merck; reagent grade, anhydrous.
8. Calgonite. Special laboratory detergent soap.
9. Cleaning solution. Formula - KH_2CrO_4 - 100 gms., H_2SO_4 (conc.) 250 ml. and 750 ml. of distilled water.
10. Crown immersion oil.
11. Carbogen (tanked). 5% carbon dioxide and 95% oxygen.

APPENDIX I.

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12. Dextrose. British Drug Houses, Limited.
Analytical reagent grade.
13. Ether. Squibb ether for anesthesia.
14. Hydrochloric acid. Decinormal.
15. Liver extract. Lederle Laboratories Division.
15 units per cc. (injectable).
16. Magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$). Eimer and
Amend. C. P. grade.
17. Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). Eimer and
Amend. C. P. grade.
18. Nembutal. Sodium ethyl (1-methyl-butyl)
barbiturate. Abbott Laboratories Ltd.
19. Penicillin. Crystalline penicillin G potassium.
Parke, Davis & Co.
20. Phenol red. B.D.H. indicator for capillator
use only. The British Drug Houses Limited.
21. Potassium chloride (KCl) Fisher Scientific Co.
C. P. grade.
22. Potassium dihydrogen phosphate. Fisher Scientific
Co. C. P. grade.
23. Silicone. General Electric Dri-film.
24. Sodium bicarbonate (NaHCO_3). The British Drug
Houses Ltd. Analytical reagent grade.

APPENDIX I.

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25. Sodium chloride (NaCl). Eimer and Amend.
C.P. grade.
26. Sodium hydroxide (NaOH). Decinormal.
27. Sodium phosphate (secondary) Na_2HPO_4 . Fisher Scientific Co. C.P. grade.
28. Toisson's fluid for red cell counts. Formula:
10 gm. NaCl, 8.0 gms. Na_2SO_4 , 30 ml. glycerine, 10 - 25
mgm. crystal violet, 160 ml. distilled water.
29. Vitamin B₁₂. Squibbs. 30 micrograms per cc. (one
microgram equivalent to 1 liver extract unit).
30. Water.
 - a. Distilled.
 - b. Double distilled. Second distilling through
all glass still.
 - c. Tap water.
31. Wright's stain. National Aniline and Chemical
Company Inc.
32. Zepherin chloride Antiseptic agent. Diluted
1:1000 with distilled water.

APPENDIX II

(i)

Introductory note:

Two values are given in the tables for each cell concentration. The first value is the number of cells per cubic millimeter and the second is the percentage value based on a 100% level at zero hours.

Group I

Bone marrow from rabbits 8 - 13 were cultured in a medium consisting of 67% Osgood's fluid and 33% homologous serum.

Rabbit No. 8

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
8A	0	11,700	100	2250	100	9450	100
	3	11,650	99.6	2700	120	8950	94.7
	7	13,100	119.7				
	12	12,650	108.1	3050	135.6	9600	101.6
	21	11,600	99.1	3425	152.2	8175	86.5
8B	0	11,025	100	2525	100	8500	100
	3	10,700	97.1	2700	106.9	8000	94.1
	7	14,025	127.2				
	12	12,550	113.8	2425	96	10,075	118.5
	21	9,900	89.8	3875	153.5	6,025	70.9

APPENDIX II

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Rabbit No. 9

Culture	Time in Hours	Total Cells %	Nucleated Cells %	Non-nucleated Cells %
9 A	0	8400 100	2275 100	6125 100
	3	11600 138.1	2350 103.3	9250 151
	8	11600 138.1	2500 109.9	9100 148.6
	12	11125 132.4	1700 74.7	9425 153.9
	24	8550 101.8	1700 74.7	6850 111.8
	27	8300 98.8	1450 63.7	6850 111.8
9 B	0	9075 100	2450 100	6625 100
	3	12400 136.6	1775 72.4	10625 160.4
	8	11250 124	1600 65.3	9650 145.7
	12	15150 166.9	2575 105.1	12575 189.8
	24	10000 110.2	1900 77.6	8100 122.3
	27	9825 108.3	2225 90.8	7600 114.7

Rabbit No. 10

10 A	0	4525 100	1475 100	3150 100
	8	5425 119.9	1400 94.9	4025 127.8
	20	5475 121	1475 100	4000 127
	24	5675 125.4	1425 96.6	4250 135
10 B	0	4725 100	1350 100	3375 100
	8	5100 107.9	1600 118.5	3500 103.7
	20	5100 107.9	2025 150	2975 88
	24	5150 109	1675 124.1	3475 103

Rabbit No. 11

11 A	0	4175 100	2875 100	1300 100
	3	5300 126.9	2025 70.4	3275 251.9
	24	4750 113.8	1650 57.4	3100 238.5
11 B	0	4700 100	2400 100	2300 100
	3	5175 110.1	2275 94.8	2900 126.1
	24	3950 84.0	1375 57.3	2575 112.

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Rabbit No. 12

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
		%		%		%	
12A	0	9775	100	3325	100	6450	100
	3	10075	103.7	3300	99.2	6775	105
	8	9200	94.1	2775	83.5	6425	99.6
	24	9250	94.6	2925	88	6325	98.1
12B	0	9200	100	3400	100	5800	100
	3	11150	121.2	4250	125	6900	119
	8	9050	98.4	3100	91.2	5950	102.6
	24	9125	99.2	2925	86	6200	106.9

Rabbit No. 13

13A	0	5475	100	2000	100	3475	100
	2	5925	108.2				
	6	5525	100.9	2450	122.5	3075	88.5
	21	6350	116	2000	100	4350	125.2
13B	0	5725	100	1825	100	3900	100
	2	5175	90.4	1950	106.8	3225	82.7
	6	6100	106.6	2500	137	3600	92.3
	21	5775	100.9	2400	131.5	3375	86.5

Group II

Bone marrow from rabbits 14, 15, 17, 18 and 19 was cultured in a medium consisting of 67% Osgood's fluid and 33% homologous serum.

Rabbit No. 14

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
		%		%		%	
14A	0	5200	100	2500	100	2700	100
	1	5325	102.4	2000	80	3325	123.1
	2	5175	99.5	1850	74	3325	123.1
	3	4925	94.7	2075	83	2850	105.6
	4	5125	98.6	1900	76	3225	119.4
	5	5525	106.3	2075	83	3450	127.8

APPENDIX II

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Rabbit No. 14 (Continued)

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
14 B	0	4025	100	1900	100	2125	100
	1	5500	136.6	1775	93.4	3725	175.3
	2	5550	137.9	1375	72.4	4175	196.5
	3	5200	129.2	1725	90.8	3475	163.5
	4	4925	122.4	2150	113.2	2775	130.6
	5						

Rabbit No. 15

15A	0	5600	100	1925	100	3675	100
	1	5250	93.8	1800	93.5	3450	93.9
	2	5850	104.5	1850	96.1	4000	108.8
	3	5050	90.2	1725	89.6	3325	90.5
	4	5575	99.6	1600	83.1	3975	108.2
	5	5225	93.3	1825	94.8	3400	92.5

15B	0	5150	100	2175	100	2975	100
	1	5075	98.5	2125	97.7	2950	99.2
	2	5925	115	1725	79.3	4200	141.2
	3	5550	107.8	1925	88.5	3625	121.8
	4	5975	116	1850	85.1	4125	138.7
	5	5550	107.8	1950	89.7	3600	121

Rabbit No. 17

17A	0	9525	100	3475	100	6025	100
	1	11550	121.3	2975	85.6	8575	142.3
	2	10825	113.6	2925	84.2	7900	131.1
	3	11375	119.4	3025	87.1	8350	138.6
	4	11425	119.9	2725	78.4	8700	144.4
	5	11450	120.2	3150	90.6	8300	137.8

17B	0	10400	100	3250	100	7150	100
	1	11150	107.2	3600	110.8	7550	105.6
	2	10575	101.7	2675	82.3	7900	110.5
	3	11800	113.5	3300	101.5	8500	118.9
	4	10800	103.8	2575	79.2	8225	115
	5	10650	102.4	3150	96.9	7500	104.9

Rabbit No. 18

18A	0	4925	100	2375	100	2550	100
	1	5000	101.5	1600	67.4	3400	133.3
	2	4950	100.5	1850	77.9	3100	121.6
	3	5325	108.1	1850	77.9	3475	136.3
	4	6350	128.9	1575	66.3	4775	187.3
	5	4900	99.5	1500	63.2	3400	133.3

APPENDIX II

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Rabbit No. 18 (Continued)

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
		%		%		%	
18B	0	6100	100	1625	100	4475	100
	1	5500	90.2	1750	107.7	3750	83.8
	2	5500	90.2	1650	101.5	3850	86.0
	3	5050	82.8	1675	103.1	3375	75.4
	4	5275	86.5	1825	112.3	3450	77.1
	5	5025	82.4	1575	96.9	3450	77.1

Rabbit No. 19

19A	0	9750	100	1800	100	7950	100
	1	10800	110.8	1625	90.3	9175	115.4
	2	10200	104.6	1850	102.8	8350	105.0
	3	10150	104.1	1675	93.1	8475	106.6
	4	10725	110	2200	122.2	8525	107.2
	5	10475	107.4	1900	105.6	8575	107.9

19B	0	9500	100	1725	100	7775	100
	1	9275	97.6	2525	146.4	6750	86.8
	2	9950	104.7	1800	104.3	8150	104.8
	3	10100	106.3	1850	107.2	8250	106.1
	4	9850	103.7	1550	89.9	8300	106.8
	5	10725	112.3	1675	97.1	9050	116.4

Group III

Bone marrow from rabbit 16 was cultured in a medium consisting of 90% Osgood's fluid and 10% homologous serum.

Rabbit No. 16

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
		%		%		%	
16A	0	6400	100	1575	100	4825	100
	1	6725	105.1	1350	85.7	5375	111.4
	2	6375	99.6	1375	87.3	5000	103.6
	3	6200	96.9	1350	85.7	4850	100.5
	4	6475	101.2	1150	73.0	5325	110.4
	5	6025	94.1	1125	71.4	4900	101.6

APPENDIX II

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Rabbit No. 16 (Continued)

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
		%		%		%	
16B	0	5725	100	1175	100	4550	100
	1	6650	116.2	1150	97.9	5500	120.9
	2	6650	116.2	1275	108.5	5375	118.1
	3	6350	110.9	1475	125.5	4875	107.1
	4	6350	110.9	1150	97.9	5200	114.3
	5	6325	110.5	1375	117	4950	108.8

Group IV

The culture medium in cultures 20A, 20B, 21A, 21B, 22A, 22B, 23A, 23B, 24A and 24B is unfortified Osgood's fluid. The corresponding C and D cultures in this group are cultured in Osgood's fluid fortified with liver extract. The concentrations of liver extract used are given at the beginning of each culture.

Rabbit No. 20

0.067 U.S.P. units of liver extract per milliliter of culture medium in cultures C and D.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
		%		%		%	
20A	0	9150	100	2425	100	6725	100
	2	6675	73	1775	73.2	4900	72.9
	4	7050	77	1100	45.4	5950	88.5
	6	5725	62.6	1125	46.4	4600	68.4
20B	0	9225	100	2525	100	6700	100
	2	7225	78.3	1750	69.3	5475	81.7
	4	8050	87.3	1850	73.3	6200	92.5
	6	6975	75.6	1375	54.5	5600	83.6
20C	0	7650	100	2025	100	5625	100
	2	8625	112.7	1975	97.5	6750	120
	4	7450	97.4	1675	82.7	5775	102.7
	6	7300	95.4	2150	106.2	5150	91.6
20D	0	7550	100	1575	100	5975	100
	2	6725	89.1	1550	98.4	5175	86.6
	4	6975	92.4	1775	112.7	5200	87
	6	6800	90.1	1575	100	5225	87.4

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Rabbit No. 21

0.67 U.S.P. units of liver extract per milliliter of
culture medium in cultures C and D.

Culture	Time in Hours	Total Cells	Nucleated Cells	Non-nucleated Cells
		%	%	%
21 A	0	8650	100	6625
	2	8800	101.7	7200
	4	7700	89	5950
	6	7000	80.9	5775
21 B	0	8700	100	6825
	2	8100	93.1	6750
	4	7350	84.5	5975
	6	7050	81	5850
21 C	0	8550	100	6825
	2	7275	85.1	5525
	4	7125	83.3	5525
	6	7775	90.9	5950
21 D	0	8050	100	6700
	2	7650	95	6325
	4	6850	85.1	5150
	6	7625	94.7	6550

Rabbit No. 22

0.0067 U.S.P. units of liver extract per milliliter of
culture medium in cultures C and D.

Culture	Time in Hours	Total Cells	Nucleated Cells	Non-nucleated Cells
		%	%	%
22 A	0	11825	100	10125
	2	11325	95.8	9750
	4	9900	83.7	8875
	6	9900	83.7	8625
22 B	0	12675	100	11150
	2	12000	94.7	10725
	4	11300	89.2	10100
	6	9800	77.3	8650
22 C	0	12300	100	10725
	2	10750	87.4	9275
	4	10700	87	9425
	6	9725	79.1	8700
22 D	0	11050	100	9050
	2	10950	99.1	9500
	4	10650	96.4	9500
	6	9600	86.9	8475

APPENDIX II

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Rabbit No. 23

0.15 U.S.P. units of liver extract per milliliter of culture medium in cultures C and D.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
23 A	0	5625	100	1350	100	4275	100
	2	5150	91.6	1100	81.5	4050	94.7
	4	5025	89.3	900	66.7	4125	96.5
	6	5700	101.3	900	66.7	4800	112.3
23 B	0	5225	100	1675	100	3550	100
	2	5200	99.5	1025	61.2	4175	117.6
	4	5150	98.6	500	29.9	4650	131
	6	5400	103.3	900	53.7	4500	126.8
23 C	0	4325	100	1325	100	3000	100
	2	4150	96	1225	92.5	2925	97.5
	4	5075	117.3	875	66	4200	140
	6	5650	130.6	1175	88.7	4475	149.2
23 D	0	3675	100	1225	100	2450	100
	2	4700	127.9	1600	130.6	3100	126.5
	4	4300	117	1350	110.2	2950	120.4
	6	4275	116.3	900	73.5	3375	137.8

Rabbit No. 24

0.15 U.S.P. units of liver extract per milliliter of culture medium in cultures C and D.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
24 A	0	4925	100	1400	100	3525	100
	2	4600	93.4	1150	82.1	3450	97.9
	4	4025	81.7	1075	76.8	2950	83.7
	6	3775	76.6	1025	73.2	2750	78
24 B	0	4850	100	1125	100	3725	100
	2	4175	86.1	1150	102.2	3025	81.2
	4	4450	91.8	1100	97.8	3350	89.9
	6	3575	73.7	1050	93.3	2525	67.8
24 C	0	4150	100	1575	100	2575	100
	2	4200	101.2	1050	66.7	3150	122.3
	4	4500	108.4	1225	77.8	3275	127.2
	6	4150	100	1175	74.6	2975	115.5
24 D	0	4450	100	1500	100	2950	100
	2	4175	93.8	1475	98.3	2700	91.5
	4	3900	87.6	1100	73.3	2800	94.9
	6	3500	78.7	1100	73.3	2400	81.4

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Group V Rat Bone Marrow

Bone marrow cells cultured in a culture medium made up of 90% Osgood's fluid and 10% homologous serum in cultures 1A, 1B, 2A and 2B.

Rat No. 1

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
1 A	0	6750	100	3525	100	3225	100
	2	4500	66.7	2425	68.8	2075	64.3
	4	5150	76.3	2350	66.7	2800	86.8
	6	6000	88.9	3025	85.8	2975	92.2
	22	4775	70.7	1950	55.3	2825	87.6
	26	4600	68.1	1175	33.3	3425	106.2
1 B	0	5625	100	2525	100	3100	100
	2	5475	97.3	2450	97	3025	97.6
	4	4825	85.8	3175	125.7	1650	53.2
	6	5575	99.1	2225	88.1	3350	108.1
	22	5225	92.9	2025	80.2	3200	103.2
	26	4375	77.8	1300	51.5	3075	99.2
Rat No. 2							
2 A	0	6750	100	2150	100	4600	100
	1½	6825	101.1	1475	68.6	5350	116.3
	3	5700	84.4	1625	75.6	4075	88.6
	5	6800	100.7	1225	57	5575	121.2
	24	4925	73	1225	57	3700	84.0
2 B	0	6625	100	1925	100	4700	100
	1½	6550	98.9	1475	76.6	5075	108
	3	5650	85.3	1375	71.4	4275	91
	5	5900	89.1	1275	66.2	4625	98.4
	24	5200	78.5	1175	61.0	4025	85.6
Rat No. 3 Bone marrow cells cultured in Osgood's fluid alone.							
3 A	0	4025	100	2150	100	1875	100
	2	4450	110.6	2075	96.5	2375	126.7
	4	3825	95	2025	94.2	1800	96
	6	3950	98.1	2225	103.5	1725	92
3 B	0	4275	100	2225	100	2025	100
	2	4275	100	2075	93.3	2200	108.6
	4	4250	99.4	1925	86.5	2325	114.8
	6	3475	81.3	1700	76.4	1775	87.7
3 C	0	4200	100	1975	100	2225	100
	2	4350	103.6	2025	102.5	2325	104.5
	4	4025	95.8	1675	84.8	2350	105.6
	6	3075	73.2	1675	84.8	1400	62.9

APPENDIX II

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Group VI Rabbit Bone Marrow Cultures (Continued).

The effect of amigen, liver extract and amigen and liver extract combined is tested in cultures from rabbits 25 - 27.

The composition of the culture medium used is given at the beginning of each culture.

Rabbit No. 25

- Culture 25A - 67% Osgood's fluid
33% homologous serum
- Culture 25B - Osgood's fluid containing
0.067 cc. amigen per cc. of Osgood's fluid.
- Culture 25C - Osgood's fluid containing
0.067 cc. amigen per cc. of Osgood's fluid
0.15 U.S.P. units of liver extract per cc.
of Osgood's fluid.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
25A	0	10950	100	3800	100	7150	100
	2	11300	103.2	3500	92.1	7800	109.1
	4	11500	105	3550	93.4	7950	111.2
25B	0	11300	100	3125	100	7175	100
	2	8550	75.7	2825	90.4	5725	79.8
	4	5150	45.6	2950	94.4	2200	30.7
25C	0	10650	100	3350	100	7300	100
	2	8875	83.3	3075	91.8	5800	79.5
	4	7975	74.9	2825	84.3	5150	70.5

Rabbit No. 26

- Culture 26A - Similar to 25A.
- Culture 26B - Osgood's fluid containing
0.01 cc. amigen per cc. of Osgood's fluid.
- Culture 26C - Osgood's fluid containing
0.01 cc. amigen per cc. of Osgood's fluid
0.15 U.S.P. units of liver extract per cc.
of Osgood's fluid.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
26A	0	12600	100	2450	100	10150	100
	2	12125	96.2	2725	111.2	9400	92.6
	4	12000	95.2	2350	95.9	9650	95
	6	12925	102.6	1975	80.6	10950	107.9

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Rabbit No. 26 (Continued)

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
26 B	0	6875	100	1775	100	5100	100
	2	5425	78.9	1125	63.4	4300	84.3
	4	5300	77.1	1100	62	4200	82.4
	6	5325	77.5	1275	71.8	4050	79.4
26 C	0	7000	100	1700	100	5300	100
	2	5775	82.5	1525	89.7	4250	80.2
	4	6700	95.7	1200	70.6	5500	103.8
	6	8000	114.3	1325	77.9	6675	125.9

Rabbit No. 27

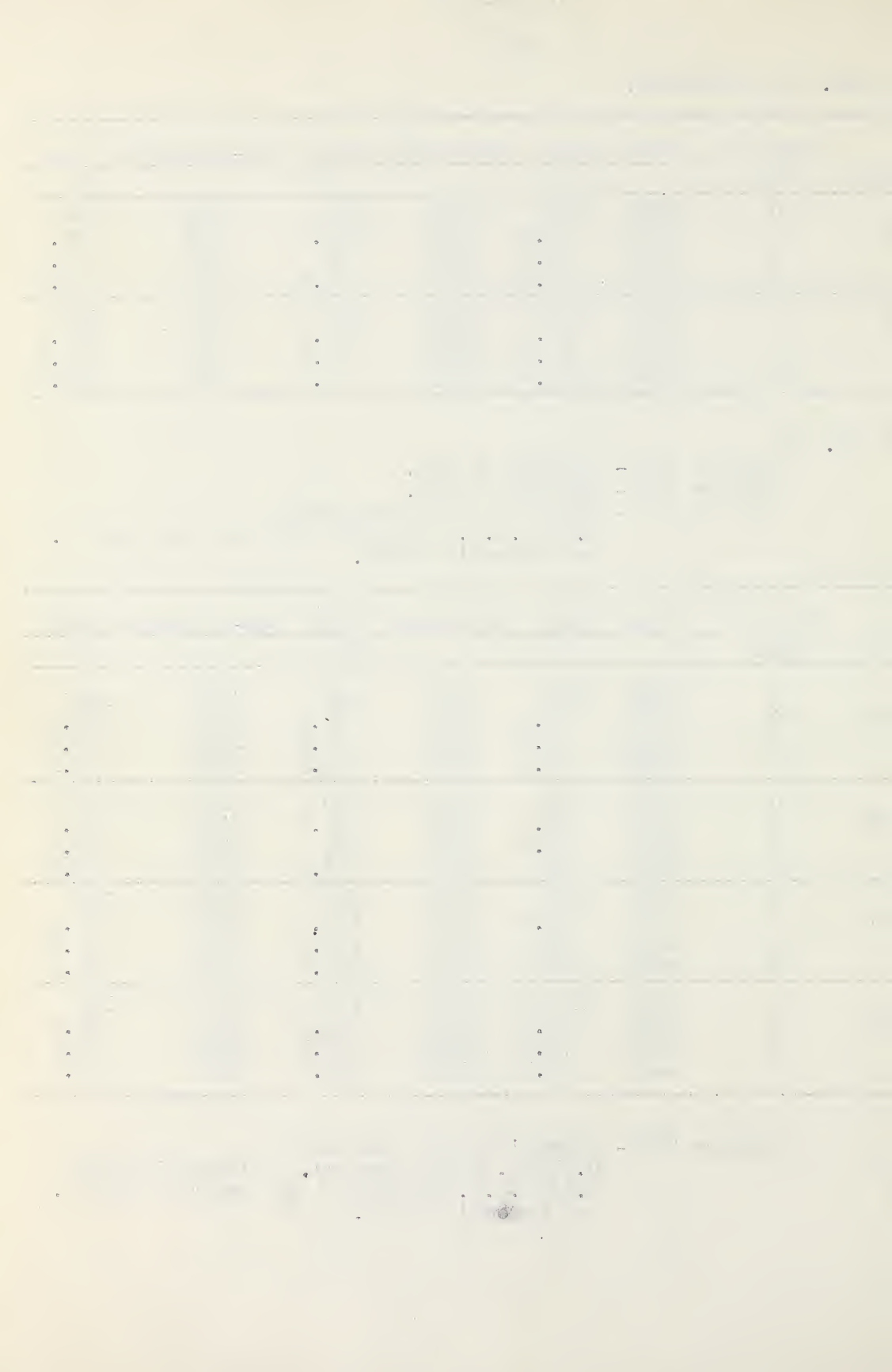
Culture 27A - Similar to 25A.

Culture 27B - Similar to 26B.

Culture 27C - Osgood's fluid containing
0.15 U.S.P. units of liver extract per cc.
of Osgood's fluid.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
27A	0	6900	100	2925	100	3975	100
	2	6400	92.7	2100	71.8	4300	108.2
	4	5400	78.3	2475	84.6	2925	73.6
	6	6100	88.4	2400	82.1	3700	93.1
27B	0	5700	100	2525	100	3175	100
	2	4925	86.4	2350	93.1	2575	81.1
	4	4825	84.6	2625	104	2200	69.3
	6	4900	86	2100	83.2	2800	88.2
27C	0	5000	100	2150	100	2850	100
	2	4925	98.5	2550	118.6	2375	83.3
	4	4450	89	2550	118.6	1900	66.7
	6	4000	80	2275	105.8	1725	60.5
27D	0	6100	100	2525	100	3575	100
	2	5500	90.2	2325	92.1	3175	88.8
	4	4850	79.5	2200	87.1	2650	74.1
	6	4400	72.1	2125	84.2	2275	63.6

Culture 27D - Osgood's fluid containing
0.01 cc. amigen per cc. of Osgood's fluid
0.15 U.S.P. units of liver extract per cc.
of Osgood's fluid.



APPENDIX II

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Group VII

A culture medium consisting of 67% Gey's solution (suitable for work under atmospheric conditions) and 33% homologous serum is employed in cultures from rabbits 28, 36, 37 and 38.

Rabbit No. 28

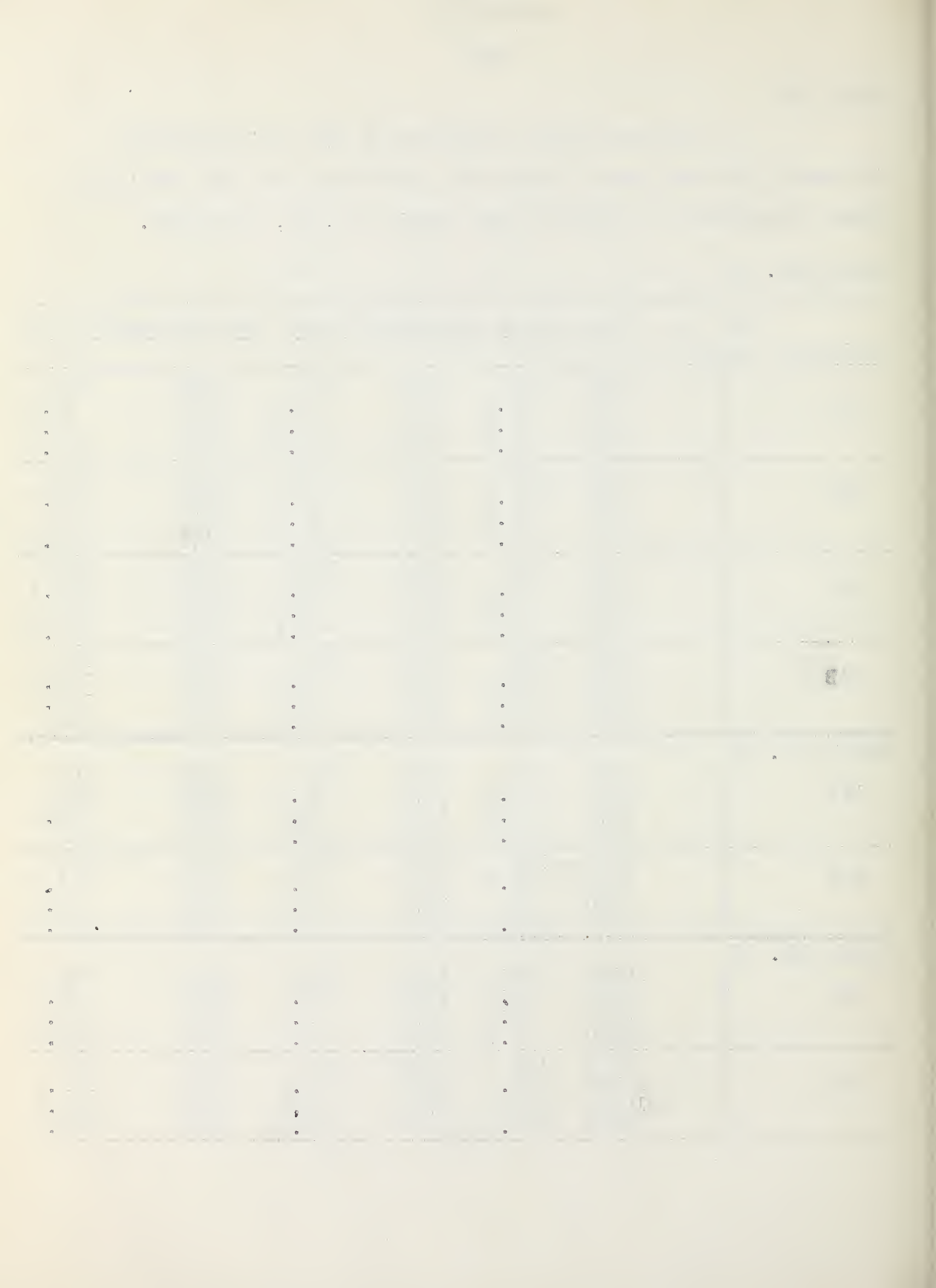
Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
28 A	0	7075	100	2575	100	4500	100
	2	6000	84.8	2425	94.2	3575	79.4
	4	6100	86.2	2625	101.9	3475	77.2
	6	6700	94.7	1875	72.8	4825	107.2
28 B	0	6250	100	2275	100	3975	100
	2	7200	115.2	2125	93.4	5025	126.4
	4	6050	96.8	2075	91.2	3975	100
	6	6775	108.4	2300	101.1	4475	112.6
28 C	0	6250	100	2750	100	3500	100
	2	5725	91.6	2075	75.5	3650	104.3
	4	6100	97.6	2425	88.2	3675	105
	6	5900	94.4	2125	77.3	3775	107.9
28 D	0	6000	100	2700	100	3300	100
	2	6400	106.7	1900	70.4	4500	136.4
	4	6200	103.3	2275	84.3	3925	118.9
	6	6400	106.7	2175	80.6	4225	128

Rabbit No. 36

36 A	0	7700	100	2700	100	5000	100
	2	9675	125.6	1475	54.6	8200	164
	4	7975	103.6	1900	70.4	6075	121.5
	6	8650	112.3	1850	68.5	6800	136
36 B	0	8400	100	2750	100	5650	100
	2	8250	98.2	1875	68.2	6375	112.8
	4	7900	94	2275	82.7	5625	99.6
	6	8675	103.3	1975	71.8	6700	118.6

Rabbit No. 37

37 A	0	10250	100	3375	100	6875	100
	2	9500	92.7	3250	96.3	6250	90.9
	4	10100	98.5	3075	91.1	7025	102.2
	6	10000	97.6	2925	86.7	7075	102.9
37 B	0	9250	100	3800	100	5450	100
	2	10100	109.2	2375	62.5	7725	141.7
	4	10450	113	2750	72.4	7700	141.3
	6	9950	107.6	3450	90.8	6500	119.3



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Rabbit No. 38

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
38 A	0	12225	100	2825	100	9400	100
	2	12025	98.4	2350	83.2	9675	102.9
	4	13950	114.1	2975	105.3	10975	116.8
	6	13975	114.3	2975	105.3	11000	117
38 B	0	12625	100	3150	100	9475	100
	2	13475	106.7	3100	98.4	10375	109.5
	4	13275	105.1	2600	82.5	10675	112.7
	6	14075	111.5	3025	96	11050	116.6

Group VIII

Cultures 29A, 29C, 31C and 31D are cultured in 67% Gey's medium and 33% homologous serum.

In cultures from rabbits 29B, 29D, 31A and 31B the culture medium is 67% Gey's medium and 33% homologous serum reinforced with liver extract. The concentrations of liver extract are given at the beginning of each table.

Rabbit No. 29

0.15 U.S.P. units of liver extract per cc. of Gey's fluid in cultures 29B and 29D.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
29 A	0	8850	100	2775	100	6075	100
	2	9550	107.9	2250	81.1	7300	120.2
	4	10025	115.8	2275	82	7750	127.6
	6	10050	119.6	1600	57.7	8450	139.1
29 B	0	9800	100	2525	100	7275	100
	2	9800	100	2125	84.2	7675	105.5
	4	10350	105.6	2000	79.2	8350	114.8
	6	9700	99	2075	82.2	7625	104.8
29 C	0	8475	100	2825	100	5650	100
	2	10300	121.5	1950	69	8350	147.8
	4	9450	111.5	2050	72.6	7400	131
	6	9625	113.6	2100	74.3	7525	133.2
29 D	0	8550	100	2650	100	5900	100
	2	10350	121.1	2350	88.7	8000	135.6
	4	9800	114.6	2150	81.1	7650	129.7
	6	9400	109.9	1800	67.9	7600	128.8

APPENDIX III

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Rabbit No. 31

0.0015 U.S.P. units of liver extract per cc. of Gey's fluid in cultures 31A and 31B.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
31A	0	8050	100	1750	100	6300	100
	2	8050	100	1800	102.9	6250	99.2
	4	8600	106.8	1950	111.4	6650	105.6
	6	9000	111.8	2100	120	6900	109.5
31B	0	7800	100	2075	100	5725	100
	2	7800	100	1575	75.9	6225	108.7
	4	7950	101.9	1550	74.7	6400	111.8
	6	8750	112.2	1675	80.7	7075	123.6
31C	0	7700	100	2100	100	5600	100
	2	8100	105.2	1575	75	6525	116.5
	4	8525	110.7	1650	78.6	6875	122.8
	6	8150	105.4	1675	79.8	6475	115.6
31D	0	8050	100	1850	100	6200	100
	2	8450	105	1800	97.3	6650	107.3
	4	8300	103.1	1575	85.1	6725	108.5
	6	9350	116.1	2200	118.9	7150	115.3

Group IX

Cultures from 30A and 30B are cultured in 67% Gey's medium and 33% foreign serum (i.e. from a second rabbit of the same species).

A similar medium reinforced with 0.0015 U.S.P. units of liver extract per milliliter of culture material is employed in cultures 30C and 30D.

Rabbit No. 30

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
30A	0	5425	100	2300	100	3175	100
	2	5475	100.9	1925	83.7	3550	111.8
	4	5025	92.6	1725	75	3300	103.9
	6	4575	84.3	950	41.3	3625	114.2
30B	0	5000	100	2250	100	2750	100
	2	5325	106.5	1950	86.7	3375	122.7
	4	5225	104.5	1350	60	3875	140.9
	6	5150	103	1525	67.8	3625	131.8

APPENDIX II

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Rabbit No. 30 (Continued)

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
30C	0	4350	100	2075	100	2275	100
	2	4800	110.3	1675	80.7	3125	137.4
	4	5450	125.3	1850	89.2	3600	158.2
	6	4650	106.9	1350	65.1	3300	145.1
30D	0	4475	100	2000	100	2475	100
	2	4825	107.8	1700	85	3125	126.3
	4	5500	122.9	2050	102.5	3450	139.4
	6	5200	116.2	1625	81.3	3575	144.4

Group X

Cultures 32A, 32C, 34C and 34D are cultured in 67%

Gey's fluid and 33% homologous serum.

Rabbit No. 32

Cultures 32B and 32D are cultured in 67% Gey's fluid and 33% human serum (from C.L.N.).

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
32A	0	7250	100	2625	100	4625	100
	2	7200	99.3	1800	68.6	5400	116.8
	4	6525	90	1950	74.3	4575	98.9
	6	7225	99.7	1875	71.4	5350	115.7
32B	0	5200	100	2775	100	2425	100
	2	2875	55.3	650	23.4	2225	91.8
	4	3725	71.6	475	17.1	3250	134
	6	2600	50	425	15.3	2175	89.7
32C	0	6075	100	2975	100	3100	100
	2	6950	114.4	1700	57.1	5250	169.4
	4	6600	108.6	1575	52.9	5025	162.1
	6	7175	118.1	2000	62.2	5175	166.9
32D	0	4800	100	2400	100	2400	100
	2	1125	23.4	850	35.4	275	11.5
	4	1000	20.8	600	25	400	16.7
	6	800	16.7	450	18.8	350	14.6

APPENDIX II

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Rabbit No. 34

Cultures from 34A and 34B are cultured in 67% Gey's fluid and 33% human serum. (from W.J.M.).

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
34A	0	3150	100	3075	100	75	100
	2	1650	52.5	1150	37.4	500	666.7
	4	1150	36.5	1100	35.8	50	66.7
	6	1700	54	900	29.3	800	1006.7
34B	0	3050	100	2900	100	150	100
	2	2000	65.6	1550	53.4	450	300
	4	2175	71.3	950	32.8	1225	816.7
	6	2825	92.6	1250	43.1	1575	1050
34C	0	12525	100	2950	100	9575	100
	2	14175	113.2	2425	82.2	11750	122.7
	4	13950	111.4	2450	83.1	11500	120.1
	6	14350	114.6	2025	68.6	12325	128.7
34D	0	14275	100	2475	100	11800	100
	2	14175	99.3	2325	93.9	11850	100.4
	4	13450	94.2	2275	91.9	11175	94.7
	6	13000	91.1	2100	84.8	10900	92.4

Group XI

Rabbit No. 33

Cultures 33A and 33B are cultured in 67% Gey's fluid and 33% homologous serum. Cultures 33C, 33D, 34E and 34F are cultured in a similar medium but which is reinforced with 0.0015 milligrams of Vitamin B₁₂ per milliliter of culture medium.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
33A	0	9850	100	3200	100	6650	100
	2	13200	134	2900	90.6	10300	154.9
	4	13300	135	2500	78.1	10800	162.4
	6	13550	137.6	1900	59.4	11650	175.2
33B	0	12550	100	3275	100	9275	100
	2	14350	114.3	2600	79.4	11750	126.7
	4	13950	111.2	2625	80.2	11325	122.1
	6	14000	111.6	2000	61.1	12000	129.4
33C	0	11200	100	3400	100	7800	100
	2	13525	120.8	3250	95.6	10275	131.7
	4	14625	130.6	2800	82.4	11825	151.6
	6	13775	123	2275	66.9	11500	147.4
33D	0	11950	100	3125	100	8825	100
	2	13100	109.6	2950	94.4	10150	115
	4	13875	116.1	2475	79.2	11400	129.2
	6	14800	123.8	2225	71.2	12575	142.5

APPENDIX II

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Rabbit No. 34

See cultures 34C and 34 D above which are controls
for 34E and 34F.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
34 E	0	12900	100	2475	100	10450	100
	2	12325	95.5	2325	93.9	10000	95.7
	4	13700	106.2	2150	86.9	11550	110.5
	6	12075	93.6	2375	96	9700	92.8
34 F	0	14725	100	2625	100	12100	100
	2	13300	90.3	2050	78.1	11250	93
	4	14350	97.5	2125	81	12225	101
	6	13100	89	2225	84.8	10875	89.9

Group XII

Rabbit No. 35

All cultures from rabbit No. 35 are cultured in 67%
Gey's fluid and 33% homologous serum.

Cultures 35C and 35D are cultured in siliconed flasks.

Culture	Time in Hours	Total Cells		Nucleated Cells		Non-nucleated Cells	
			%		%		%
35 A	0	14525	100	1900	100	12625	100
	2	16275	112	1600	84.2	14675	116.2
	4	16450	113.3	1350	71.1	15100	119.6
	6	16350	112.6	1350	71.1	15000	118.8
35 B	0	15350	100	2200	100	13150	100
	2	16350	106.5	1850	84.1	14500	110.3
	4	15650	102	1825	83	13825	105.1
	6	17300	112.7	1525	69.3	15775	120
35 C	0	15000	100	2150	100	12850	100
	2	14925	99.5	1725	80.2	13200	102.7
	4	15650	104.3	1600	74.4	14050	109.3
	6	17225	114.8	1825	84.9	15400	119.8
35 D	0	14800	100	1875	100	12925	100
	2	17200	116.2	1900	101.3	15300	118.4
	4	17175	116	1650	88	15525	120.1
	6	17750	119.9	1600	85.3	16150	125

